A calorimetric study has been made of the interaction between the lac repressor and isopropyl-1-thio-β-D-galactopyranoside (IPTG). The buffer-corrected enthalpy of reaction at 25 °C was found to be -15.6, -24.7, -4.6 kJ/mol of bound IPTG at pH 7.0, pH 8.1, and pH 9.0, respectively. This large range of enthalpy values is in contrast to a maximum difference in the free energy of the reaction of only 1.5 kJ/mol of bound IPTG between these pH values. The reaction was found by calorimetric measurements in different buffers to be accompanied by an uptake of 0.29 mol of protons/mol of bound IPTG at pH 8.1. The pH dependency of the reaction enthalpy suggests differences in the extent of protonation of the binding site and the involvement of H bonding with IPTG.

The lack of strong hydrophobic contributions in the IPTG binding process is revealed by the absence of any determinable heat capacity change for the reaction at pH 7.0.

The presence of phosphate buffer significantly alters the enthalpy of IPTG binding at higher pH values, but has little effect upon the binding constant. This implies that highly negative phosphate species change the nature of the IPTG binding site without any displacement of phosphate upon IPTG binding.

The Escherichia coli lactose operon is under negative control of the lac repressor protein which binds specifically to the operator portion of the E. coli genome. In vivo, the interaction between the repressor protein and the operator is modulated by an inducer, allolactose (Jobe and Bourgeois, 1972). A number of kinetic and equilibrium physical chemical methods has been applied in vitro to determine the binding constants for the interaction of various synthetic inducers with the repressor protein as well as the number of binding sites for these inducers on the repressor which is a tetrameric protein (for recent reviews, see Barkley and Bourgeois, 1978, and von Hippel, 1979). The effect of temperature, pH, ionic strength, and temperature.

The action of the inducer was imagined to cause an increase in the population of the conformation of the repressor protein which does not bind to the operator region. This allosteric mechanism in its simplest form has a necessary consequence of cooperative inducer binding, yet no such cooperativity had been clearly observed in IPTG binding studies of the repressor in the absence of operator.

This situation has been clarified by O'Gorman et al. (1980) who found signs of positive cooperativity for the binding of IPTG to the repressor protein in the presence of a 29-base pair operator DNA fragment using fluorescence spectroscopy to determine the extent of IPTG binding at pH 7.5. Assuming that the repressor tetramer has two binding sites for the operator and four for IPTG, they could fit their data to the Monod-Wyman-Changeux model (Monod et al., 1965). However, in the absence of the operator fragment, no cooperativity is observed. The presence of the operator is required to cause a shift in the repressor population to the T form which has a lower affinity for IPTG. The presence of IPTG with its higher affinity to the R form gives rise to an increase in the amount of R form repressor and gives rise to the observation of positive cooperativity.

In view of the general importance of this regulatory protein, we were interested in gaining further thermodynamic information to aid in the interpretation of the nature of the interaction between IPTG and the repressor protein. The free energy of IPTG binding is obtainable from the binding studies mentioned above. By measuring the heat of reaction, the change of the entropy of the IPTG binding reaction can be assessed, and the relative importance of enthalpy and entropy contributions to the free energy can then be delineated. Extending the heats of reaction studies to various temperatures, the heat capacity change can be evaluated. These thermodynamic parameters might reflect any unusual energetic or hydrophobic features of the IPTG binding to the repressor protein. Heat of reaction determined under different pH conditions could also reveal the importance of protons in the binding process. Although reasonable quantities of repressor can be obtained for selected calorimetric studies, sufficient amounts of operator fragments are not yet available for our purposes. Consequently, our initial efforts have been limited to IPTG binding to repressor in the absence of operator. We have made use of a recently developed titrating calorimeter designed for the use of nanomolar amounts of reactants (Spokane and Gill, 1981) which are compatible with the quantity of repressor that is available with reasonable purification efforts. Further miniaturization of calorimetric techniques along with enhanced production of operator fragments should allow extension of the present investigations.

MATERIALS AND METHODS

Chemicals—Diisopropyl fluorophosphate, IPTG, and tetracycline hydrochloride were bought from Sigma. ¹⁴C-labeled IPTG was ob-
Repressor Preparation—A DH5α(pH1Q6) strain derived from E. coli K12 which contained a pMB9 plasmid carrying the lac I gene (Hare and Sadler, 1978) was used as the repressor source (the gift of these from Drs. J. L. Betz and J. R. Sadler is gratefully acknowledged). The cells were grown in SLBH broth as described by Betz and Sadler (1978) with tetracycline at a concentration of 10 mg/liter until the A525 = 10. The cells were then harvested and stored at -70°C until the repressor was to be extracted.

We employed the method of Rosenberg et al. (1977) for the extraction and purification of the lac repressor from these cells. However, the Sephadex G-200 step and use of ortho-toluenesulfonic acid were omitted. The repressor protein was eluted from the phosphocellulose column with a 0.12-0.35 M phosphate gradient. The purity was monitored by the ammonium sulfate precipitation method with 1°C-labeled IPTG as described by Jabe et al. (1972). Those fractions from the phosphocellulose column which were used were pooled and precipitated with ammonium sulfate to 0.5 a and pelleted by centrifugation, whereupon the pellet was stored at -70°C. Four different preparations of lac repressor were used in this study and all of them were >95% pure as judged by disaccharide sodium dodecyl sulfate-gel electrophoresis in 11% polyacrylamide according to Neville (1971).

Calorimetry.—The calorimeter used in this study is a titration microcalorimeter designed to allow measurements on nanomolar concentrations of repressor subunits. A final

\[ R, = R, (1 - \frac{v}{V}) \]

\[ L, = L, (1 - (1 - \frac{v}{V}) \]

where \( R, \) is the initial concentration of repressor binding sites, \( L, \) is the concentration of IPTG in the syringe, \( R, \) and \( L, \) are the total concentrations of repressor binding sites and IPTG, respectively, in the reaction cell after the \( i \)th injection, \( v, \) is the injection volume, \( V, \) is the volume of the reaction cell, and \( i \) is the injection number. The heat/injection is determined by integration of the power into the calorimeter.

Repressor solutions were prepared fresh for each experiment by weighing in a sample of the stored ammonium sulfate precipitate, dissolving it in the buffer to be used for the experiment, and dialyzing it overnight against two samples of 1 liter each of the same buffer. After centrifugation at 45,000 x g for 30 min, the absorbance at 280 and 340 nm was determined and the solutions diluted with dialysate to be typically 8 x 10^-4 M with respect to repressor subunits. A final absorbance reading was made and the repressor solution loaded into the reaction cell of the microcalorimeter. The repressor subunit concentration was determined using \( \varepsilon_{280} = 2.25 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \); and none of the solutions had an absorbance at 340 nm exceeding 2% of the value at 280 nm (Butler et al., 1977).

The volume/IPTG injection was typically 10.64 μl, and the reaction heat was measured in a period of approximately 5 min from the start of each injection. A series of 11-20 injections was used to titrate the repressor solution. The thermal titrations were performed at 24.95 or 14.95°C. All solutions were 0.5 M with respect to potassium chloride. Potassium phosphate, Tris, and bis-tris, respectively, were used at a 0.1 M concentration to buffer the solutions at pH 7.0, 8.1, and 9.0. In one series of experiments, the phosphate concentration was changed to 0.5 and 0.01 M at pH 7.0.

RESULTS AND DISCUSSION

Fig. 1 shows how the measured heat/mol of injected IPTG varies with the number of injections as a solution of lac repressor is titrated with an IPTG solution. During the first injections, the repressor is at an excess and most of the added IPTG is reacted with the repressor so that the measured heat for each injection, \( Q_m \), is dominated by the heat of reaction, \( Q_R \). Once the repressor is saturated, \( Q_M \) becomes a measure of heats of dilution of IPTG and the repressor as well as heats of transfer due to thermal inhomogeneities in the calorimeter. These cumulative heat changes are designated by \( Q_0 \) and once determined may be subtracted from \( Q_M \) to isolate the heat of reaction.

A simple graphical estimate of the stoichiometry of the reaction can be obtained by extrapolating the ends of a plot (Fig. 2) of the cumulative \( Q_M \) as a function of the ratio between the concentration of IPTG and repressor subunits after each injection (see below). For the experiments described in this paper, the stoichiometry determined graphically was found to be 1 mol of IPTG/mol of repressor subunit.

In order to determine the enthalpy change/mol of reaction, \( \Delta H^\circ \), and the dissociation constant, \( K_D \), for the binding of IPTG to the lac repressor, a nonlinear least square fit using the Marquardt algorithm (Marquardt, 1963) was employed. As outlined above,

\[ Q_M = Q_T \]

\[ S = \frac{Q_M}{Q_T} \]

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where \( \theta \) is the fraction of repressor binding sites occupied by IPTG after the \( i \)th injection and \( n_i \) is the total amount of repressor binding sites after the \( i \)th injection. \( \theta \) depends on

\[ \theta = \frac{Q_M}{Q_T} \]
the binding equation describing the reaction. Our graphical determination of the stoichiometry invariably pointed to the binding of 1 mol of IPTG/mol of repressor subunits.

The absence of cooperative behavior of IPTG binding (as shown by O’Gorman et al., 1980) under the experimental condition used in this work suggests that IPTG binds independently to one binding site/subunit, all of which are identical. For this simple situation, the fraction of binding at the ith titration step is given by the following equation.

\[ \theta_i = \frac{R_i + L_i + K_D - (R_i + L_i + 3) - 4RL_i}{2R_i} \]  

Table I presents the results of this data fitting procedure as applied to heat values from experiments conducted at different pH values and in the presence of different buffer ions. The standard error of the difference between the measured injection heat values and those derived from the use of the best fit parameters is in all cases within a factor of two of the standard error of the calorimetric base-line fluctuations. Thus, the simple identical site model is found to be consistent with the precision of our data.

Two things are noteworthy in Table I. First, at pH 7.0 and 9.0, the \( \Delta H^o \) for the reaction is insensitive to the presence of either Tris or bis-tris. That means that neither of these two buffer ions interact with the repressor or, if they do, there is no change in the interaction when IPTG is bound. It also suggests that there is no change in protonation upon IPTG binding as is also implied by the lack of pH dependence of \( K_D \).

![Graph showing the cumulative heats/injection as a function of the molar ratio of IPTG and repressor subunits found after each injection.](image)

**Figure 2.** The cumulative heats/injection as a function of the molar ratio of IPTG and repressor subunits found after each injection. See the experiment as described in the legend to Fig. 1. The stoichiometry of the reaction is determined from the intersection of the asymptotes (broken lines) of the curve.

**Table I**

The enthalpy of reaction and the dissociation constant for the binding of IPTG to the lac repressor at different pH values

| pH  | Buffer  | \( \Delta H^o \) kJ/mol bound IPTG | \( 10^9 \times K_D \) M |
|-----|---------|-----------------------------------|--------------------------|
| 7.0 | Tris    | -16.1                             | 2.6                      |
| 7.0 | Bis-tris| -15.0                             | 5.4                      |
| 7.0 | Phosphate| -17.0                             | 2.6                      |
| 8.1 | Tris    | -11.0                             | 4.1                      |
| 8.1 | Bis-tris| -16.6                             | 7.0                      |
| 8.1 | Phosphate| -16.1                             | 3.0                      |
| 9.0 | Tris    | -4.6                              | 7.4                      |
| 9.0 | Bis-tris| -4.6                              | 7.4                      |
| 9.0 | Phosphate| -32.2                             | 5.5                      |

*This value was taken from the bis-tris experiment at the same pH and fixed in the fitting procedure described under “Results and Discussion” so as to get the best fit.

At these pH values from the results of O’Gorman et al. (1980). At the intermediate pH 8.1, however, there is a difference between the \( \Delta H^o \) values of 5.6 kJ/mol when Tris or bis-tris is used. Since the heat of proton dissociation of bis-tris is 28.2 kJ/mol (Paabo and Bates, 1970) and of Tris is 47.6 kJ/mol (Christensen et al., 1980), at 25 °C, this difference cannot be explained by an uptake of 0.29 mol of protons/mol of bound IPTG. This gives a buffer corrected \( \Delta H^o = -24.7 \) kJ/mol of bound IPTG at pH 8.1. Butler et al. (1977) found at pH 7.6 that \( \Delta H^o = -25.9 \) kJ/mol, a value derived from a van’t Hoff plot using the \( \Delta G^o \) values at 4 and 25 °C. The expected value should be the sum of the heat of protonation of basic groups in the repressor and an intrinsic IPTG binding heat value of -10 kJ/mol (the average \( \Delta H^o \) values for the binding of IPTG in the presence of bis-tris and Tris at pH 7.0 and 9.0, where linked protonation is absent). Thus, the heat of IPTG-linked protonation of basic groups in the repressor would be approximately -15 kJ/mol of IPTG bound, i.e. -45 kJ/mol of protons. This value agrees with heats of protonation of the side chains of free lysine and arginine (Christensen et al., 1976).

In order to examine the importance of contributing thermodynamic factors into the energetics of IPTG binding, we utilized the enthalpy of reaction values in conjunction with equilibrium constant determinations. From the average of \( K_D \) values for the Tris and bis-tris experiments, the free energy change, \( \Delta G^o \), for the binding of IPTG to the repressor binding sites is -30.8 kJ/mol at pH 7.0 and -29.3 kJ/mol at pH 9.0 with a standard state of 1 M. The associated change in entropy, \( \Delta S^o \), using \( \Delta S^o = (\Delta H^o - \Delta G^o)/T_\text{R} \), is 51.0 J/K·mol at pH 7.0 and 82.9 J/K·mol at pH 9.0. If \( \Delta S^o \) is based on mole fractions instead of moles/liter, the sign changes so that the value becomes -2.76 kJ/K at pH 7.0 and -0.72 kJ/K at pH 9.0. These results suggest that the binding of IPTG at these pH values involves little hydrophobic interaction. This is confirmed more directly by the lack of significant \( \Delta C_p^o \) from measurements of \( \Delta H^o = -14.1 \) kJ/mol at 14.95 °C in Tris at pH 7.0.

The deceitful lack of major difference between \( \Delta G^o \) for the binding of IPTG at pH 7.0 and 9.0 agrees well with values found in the earlier literature (Barley and Bourgeois, 1978). It is contrasted by the strikingly large differences found for \( \Delta H^o \) values at these pH values. Thus, despite the similar \( \Delta G^o \) values, the intrinsic binding site for IPTG on the repressor must be quite different at pH 7.0 and 9.0, most likely due to differences in the extent of protonation of the binding site at the two pH values. Presumably at the low pH value (7.0), the protonated site interacts with H bond formation to IPTG to give a significantly more exothermic reaction.

The calorimetric results obtained in the presence of phosphate buffer (Table I) indicate that a complex role is played by this buffer in its effect upon IPTG binding. Curiously, no significant effect was found upon the binding constant at all pH values studied. Even at 0.5 M potassium phosphate (pH 7.0), no noticeable effect was observed on \( K_D \). However, at pH 8.1 where \( \Delta H^o = -16.1 \) kJ/mol of IPTG in phosphate buffer, compared to buffer-corrected heat (Tris and bis-tris) of -25 kJ/mol of IPTG, the presence of phosphate contributes nearly +9 kJ/mol to the IPTG binding process. Moreover, at pH 9.0 where a surprising value of -92 kJ/mol of IPTG in the presence of phosphate was found in comparison with -5 kJ/mol of IPTG in Tris and bis-tris buffers, the presence of phosphate contributes nearly +27 kJ/mol to the binding enthalpy, when compared to tris buffer. These facts suggest that the phosphate significantly alters the IPTG binding site in so far as the enthalpy and entropy change of the reaction are concerned but leaves the free energy change of IPTG binding unchanged.
unaffected. Furthermore, the bound phosphate must not be displaced upon IPTG binding under the solution conditions employed since the phosphate effect on \( K_D \) is negligible. The large enthalpy effects at high pH suggests that highly negatively charged phosphate species are involved in modifying the nature of the IPTG binding site.

Overall, these results show that IPTG binding to repressor in the absence of operator is characterized by a reaction to identical equivalent sites without any implication of allosteric transition involved in the process. The energetics of the binding process suggests the absence of hydrophobic interactions and the likely presence of nonionic hydrogen bonding effects.

REFERENCES

Barkley, M. D., and Bourgeois, S. (1976) in The Operon (Mill, J. S., and Reznikoff, W. S., eds) pp. 177-220, Cold Spring Harbor Laboratory

Betz, J. L., and Sadler, J. R. (1976) J. Mol. Biol. 105, 293-319

Butler, A. P., Revzin, A., and von Hippel, P. H. (1977) Biochemistry 16, 4757-4768

Christensen, J. J., Hansen, D., and Izatt, R. M. (1976) Handbook of Proton Ionization Heats and Related Thermodynamic Quantities John Wiley and Sons, New York

Christensen, J. J., Wrathall, D. P., and Izatt, R. M. (1968) Anal. Chem. 40, 175-181

Hare, D. L., and Sadler, J. R. (1978) Gene 3, 269-278

Jobe, A., and Bourgeois, S. (1972) J. Mol. Biol. 68, 397-408

Jobe, A., Riggs, A. D., and Bourgeois, S. (1972) J. Mol. Biol. 64, 181-199

Marquardt, D. W. (1963) J. Soc. Ind. Appl. Math. 11, 431-441

Monod, J., Wyman, J., and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88-118

Neville, D. M. (1971) J. Biol. Chem. 246, 6328-6334

O’Gorman, R. B., Rosenberg, J. M., Kallai, O. B., Dickerson, R. E., Itakura, K., Riggs, A. D., and Matthews, K. S. (1980) J. Biol. Chem. 255, 10107-10114

Paabo, M., and Bates, R. G. (1970) J. Phys. Chem. 74, 702-705

Rosenberg, J. M., Kallai, O. B., Kopka, M. L., Dickerson, R. E., and Riggs, A. D. (1977) Nucleic Acids Res. 4, 567-572

Spokane, R. B., and Gill, S. J. (1981) Rev. Sci. Instrum. 52, 1728-1733

von Hippel, P. H. (1979) in Biological Regulation and Development: Gene Expression (Goldberger, R. F., ed) Vol. 1., pp. 279-348, Plenum Press, New York