Allosteric Regulation of Inducer and Operator Binding to the Lactose Repressor†

Thomas J. Daly and Kathleen Shive Matthews*
Department of Biochemistry, Rice University, Houston, Texas 77251
Received December 18, 1985; Revised Manuscript Received May 2, 1986

ABSTRACT: The effects of cysteine modification and variations in pH on the equilibrium parameters for inducer and operator binding to the lactose repressor protein were examined. Operator binding affinity was minimally affected by increasing the pH from 7.5 to 9.2, whereas inducer binding was decreased for both the unliganded protein and the repressor–operator complex over the same range. Inducer binding to the repressor became more cooperative at high pH. The midpoint for the change in inducer affinity and cooperativity was pH 8.3; this value correlates well with cysteine ionization. The differential between repressor–operator affinity in the presence and absence of inducer was significantly decreased by modification of the protein with methyl methanethiosulfonate (MMTS). In contrast to unreacted protein, the inducer binding parameters for MMTS-modified repressor were largely unaffected by pH variation. The free energy for formation of the completely liganded protein was calculated for two pathways; the ΔG values for these two independent routes were equivalent only for stoichiometries of four inducers and two operators per repressor molecule. All of the binding data were analyzed quantitatively by using a Monod–Wyman–Changeux two-state model for allosteric regulation. The observed dependences of the isopropyl β-D-thiogalactoside binding curves on pH, DNA concentration, and MMTS modification were fitted by varying only the equilibrium constant between the two conformational states of the protein. With this analysis, high pH favors the T (high operator/low inducer affinity) state, while modification of cysteine-281 with MMTS elicits a shift into the R (high inducer/low operator affinity) state. These results suggest strongly that ionization or covalent modification of cysteine-281 markedly influences the quaternary conformational equilibrium of tetrameric repressor molecules.

Regulating transcription of mRNA coding for the lactose metabolizing enzymes in *Escherichia coli* is dependent upon alteration of the operator binding affinity of the lactose repressor protein by inducer sugars as environmental conditions vary (Miller & Reznikoff, 1980). In the presence of operator DNA, cooperative inducer binding to the repressor has been observed (O'Gorman et al., 1980). As with many multimeric proteins, cooperativity toward ligand binding suggests allosteric regulation of protein function (Monod et al., 1965). The monomeric mutant repressor (T-41) resulting from a mutation at tyrosine-282 (Schmitz et al., 1976; Ganem et al., 1973) exhibits wild-type inducer binding characteristics at pH 7.5 but no pH dependence for either inducer binding affinity or inducer binding rates (Daly & Matthews, 1986). Quaternary structure, therefore, is required for pH-associated effects on inducer binding. Modification of the lactose repressor at cysteine-281 with methyl methanethiosulfonate (MMTS)[1] correlates with decreased inducer binding rates and decreased operator binding affinity (Daly et al., 1986). The effects of MMTS modification at Cys-281 and the observation that loss of quaternary structure, concomitant with replacement of tyrosine-282 with a serine residue, abrogated the pH-associated changes in inducer binding parameters suggested that this region of the protein was important in regulating repressor function. We have utilized chemical modification of Cys-281 and pH variation as means to explore changes in the equilibrium binding parameters for the repressor protein.

Materials and Methods

Buffer. TMS buffer consisting of 0.01 M Tris-HCl, 0.001 M EDTA, 0.01 M $MgCl_2$, and 0.2 M KCl was used for all of the binding assays as well as the kinetic experiments. Only the pH of the TMS buffer was varied as noted.

Isolation of Protein. Lactose repressor protein was isolated from *Escherichia coli* CSH 46 as described (Rosenberg et al., 1977; O'Gorman et al., 1980). Samples of frozen repressor were thawed for use and dialyzed against 0.1 M Tris-HCl, pH 7.5, or against TMS buffer. Unless noted, wild-type or T-41 mutant repressor, dialyzed against 0.1 M Tris-HCl, pH 7.5, was modified with a 150-fold molar ratio of MMTS over repressor monomer for 30 min at 4 °C. Following reaction, excess MMTS was removed by dialysis against TMS buffer. T-41 mutant repressor was purified as described previously (Daly & Matthews, 1986). Purity of all protein species was >95% as determined by SDS gel electrophoresis.

Assay of Activity. Operator DNA binding in the absence and presence of inducer (2×10^{-3} M) was measured by the nitrocellulose filter binding assay of Riggs et al. (1968) with several modifications. λp*lac* DNA was replaced by 40 base pair operator DNA isolated from plasmid pOE101 (Lillis et al., 1982). The operator fragment was labeled with [^{32}P]-dαATP and [^{32}P]dαTTP by using DNA polymerase large fragment (Klenow). The incubation buffer used in the binding assays was TMS with 50 μg/mL BSA. Operator concentration was $\sim 8 \times 10^{-12}$ M. Following filtration of incubated samples, nitrocellulose filters were not washed to avoid differences in retention due to changes in dissociation rates. Filters were then dried and counted in scintillation fluid. Background counts were subtracted to generate saturation curves. Operator affinity was determined from the reciprocal

† This work was supported by grants from the National Institutes of Health (GM 22441) and the Robert A. Welch Foundation (C-576). T.J.D. was a National Institutes of Health Trainee (GM 07833).
* Correspondence should be addressed to this author.

[1] Abbreviations: BNP, 2-(bromoacetamido)-4-nitrophenol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; MMTS, methyl methanethiosulfonate; MNP, 2-(chloromercuri)-4-nitrophenol; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; bp, base pair(s).

0006-2960/86/0425-5479$01.50/0

Table I: Apparent Equilibrium Association Constants

condition	repressor + operator[a] ($M^{-1} \times 10^{-9}$)	repressor–inducer + operator[a] ($M^{-1} \times 10^{-6}$)	repressor + inducer[b] ($M^{-1} \times 10^{-5}$)	repressor–operator + inducer[b] ($M^{-1} \times 10^{-5}$)	L[c]
wild-type					
pH 7.5	1.6 (±0.4)	6.3 (±0.9)	7.1 (±0.9)	0.40 (±0.016)	1
pH 9.2	1.7 (±0.3)	6.4 (±1.4)	1.0 (±0.2)	0.077 (±0.006)	1×10^{4}
MMTS, pH 7.5	0.038 (±0.01)	7.9 (±1.5)	7.2 (±0.9)	4.6 (±0.2)	1×10^{-4}
MMTS, pH 9.2	0.079 (±0.006)	8.8 (±1.6)	5.9 (±0.3)	1.1 (±0.3)	3×10^{-2}
T-41 monomer					
pH 7.5			8.3 (±0.9)		
pH 9.2			5.9 (±0.5)		
MMTS reacted, pH 7.5			5.0 (±0.6)		

[a] Reciprocal of the repressor concentration at 50% saturation. [b] Reciprocal of the inducer concentration at 50% saturation. [c] $L = T/R$.

of the repressor concentration at half-saturation. Saturation levels were measured directly at excess repressor concentrations and confirmed by double reciprocal plots of the data.

Inducer binding activities were measured by IPTG titration of protein (10^{-7} M), monitoring tryptophan fluorescence emission intensity changes (Daly et al., 1986). Apparent inducer affinity was determined from the reciprocal of the IPTG concentration at half-saturation. Inducer binding to repressor–operator complex was similarly assayed except that 40 bp operator DNA was present (2×10^{-6} M). Operator DNA concentrations were determined by the ethidium bromide fluorescence assay of LePecq and Paoletti (1966). All inducer titration assays were performed in TMS buffer of specified pH at ambient temperature (23–25 °C).

Predicted Curves for Monod–Wyman–Changeux Modeling. Theoretical curves simulating allosteric inducer binding were generated on a Macintosh personal computer using a modeling program adapted by George Herrin. Modeling was based upon a two operator binding site scheme as previously described (O'Gorman et al., 1980).

Results

Effects of High pH and MMTS Modification on Operator DNA Binding Constants. Equilibrium association constants for operator in the presence and absence of inducer were determined for MMTS-modified and unmodified repressor at pH 7.5 and 9.2 (Table I). In the presence of 0.2 M KCl and 0.01 M $MgCl_2$ (TMS buffer), the operator equilibrium constant for unmodified repressor exhibited no pH dependence. The operator affinity for repressor modified with MMTS was decreased ~40-fold compared to unreacted protein, whereas when IPTG (2×10^{-3} M) was bound the affinity of the modified protein for operator was similar to that of induced native protein. The differential between operator DNA binding affinity in the absence and presence of inducer was decreased for the MMTS-modified repressor at both pH values. The affinity of MMTS-modified repressor for operator at pH 9.2, although diminished compared to unreacted protein, was 2-fold greater than that observed for MMTS–repressor at pH 7.5. The similarity of the K_a values for operator in the presence of inducer under all conditions indicates that the effects of pH and MMTS modification cannot be ascribed to nonspecific alterations in the protein–DNA interaction but rather are due to specific changes which affect binding to the operator sequence.

Effect of High pH and Cysteine Modification on Inducer Binding Constants. Inducer binding to MMTS-modified and unmodified repressor was monitored as the fraction (Y) of the total change in fluorescence at increasing inducer concentrations (Figure 1A). The apparent inducer affinities were similar at pH 7.5 for repressor and MMTS-modified protein (Table I). The inducer affinity for MMTS-modified repressor at high pH was only slightly diminished; however, the affinity of unmodified repressor for inducer was decreased ~7-fold at pH 9.2. Hill plots derived from the inducer titration data in Figure 1A are shown in Figure 1B. Significant cooperativity, reflected in a Hill coefficient of 1.9, was observed only for unmodified repressor at pH 9.2; curves for the remainder of the proteins yielded Hill coefficients ≤1.1.

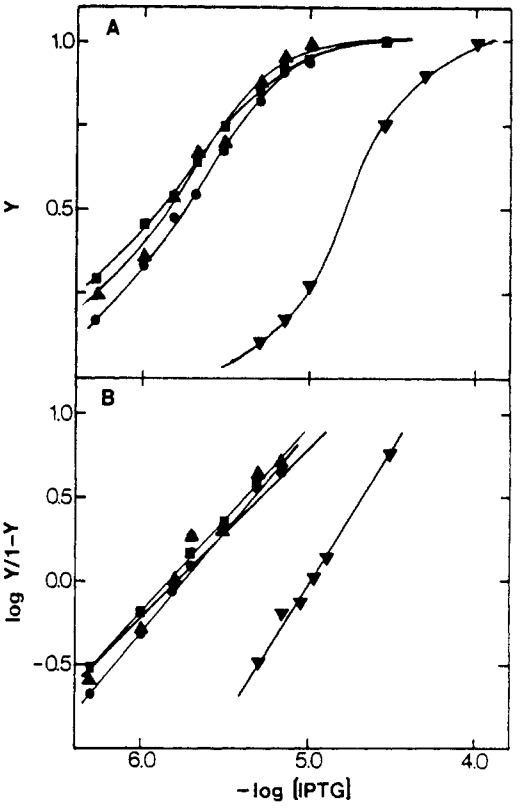
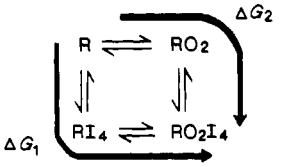


FIGURE 1: pH effects on inducer binding to MMTS-modified and unmodified repressor. Concentration of protein was 1×10^{-7} M. All titrations were performed in buffer containing 0.01 M Tris-HCl, 0.001 M EDTA, 0.01 M $MgCl_2$, and 0.2 M KCl. Fluorescence excitation was at 285 nm, with fluorescence emission monitored at wavelengths above 350 nm. (▲) Unmodified repressor, pH 7.5; (▼) unmodified repressor, pH 9.2; (■) MMTS-modified repressor, pH 7.5; (●) MMTS-modified repressor, pH 9.2. (A) Fraction of the total change in fluorescence vs. log IPTG concentration. (B) Hill plot of data shown in (A).

Inducer titrations of MMTS-modified and unmodified repressor were executed in the presence of saturating concen-

Table II: Free Energy of Ligand Binding to Repressor[a]



	$\Delta G_{R/I}$, repressor + inducer (kcal)	$\Delta G_{RO/I}$, repressor–operator + inducer (kcal)	$\Delta G_{R/O}$, repressor + operator (kcal)	$\Delta G_{RI/O}$, repressor–inducer + operator (kcal)	ΔG_1[b] (kcal)	ΔG_2[c] (kcal)
pH 7.5	−7.95	−6.25	−12.48	−9.23	−50.3	−50.0
pH 9.2	−6.79	−5.28	−12.53	−9.23	−45.6	−46.2
MMTS reacted, pH 7.5	−7.95	−7.69	−10.28	−9.36	−50.5	−51.3
MMTS reacted, pH 9.2	−7.84	−6.87	−10.76	−9.45	−50.3	−49.0

[a] $\Delta G = -RT \ln K_a$, T = 298 K. IPTG, n = 4; operator, n = 2. [b] $\Delta G_1 = 4\Delta G_{R/I} + 2\Delta G_{RI/O}$. [c] $\Delta G_2 = 2\Delta G_{R/O} + 4\Delta G_{RO/I}$.

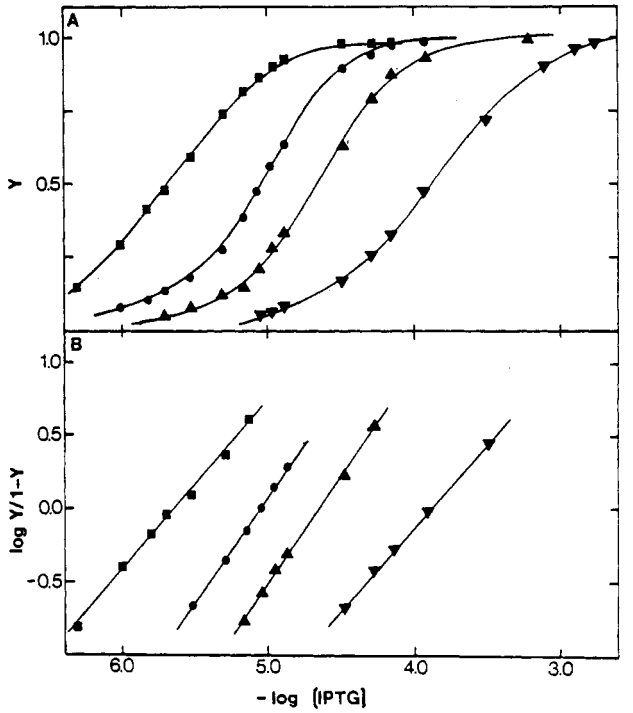


FIGURE 2: pH effects on inducer binding to unmodified and MMTS-modified repressor in the presence of operator DNA. Titrations were performed as described under Materials and Methods and Figure 1. Operator DNA was present at 2×10^{-6} M; this concentration of operator is sufficiently high to maintain association with repressor in the presence of saturating inducer concentrations. (▲) Unmodified repressor, pH 7.5; (▼) unmodified repressor, pH 9.2; (■) MMTS-modified repressor, pH 7.5; (●) MMTS-modified repressor, pH 9.2. (A) Fraction of the total fluorescence change vs. log IPTG concentration. (B) Hill plot of data shown in (A).

trations of operator DNA (Figure 2A). The apparent inducer affinity differed for each of the protein samples (Table I). No cooperativity (Hill coefficient ≤1.1) was observed for unmodified repressor at pH 9.2 or for MMTS-modified repressor at pH 7.5 (Figure 2B). In contrast, Hill coefficients of ~1.4 were determined for the operator complexes of unmodified repressor at pH 7.5 and MMTS-modified repressor at pH 9.2; this value agrees with the results previously obtained for the unreacted protein near neutral pH (O'Gorman et al., 1980). The pH dependence of inducer binding to monomeric mutant repressor was compared to wild-type unmodified repressor. As shown in Table I, minimal change in the affinity of inducer for mutant monomer was observed with increasing pH. As anticipated, the monomeric mutant repressor exhibited no cooperativity at any pH (Hill coefficient = 1.0), in contrast

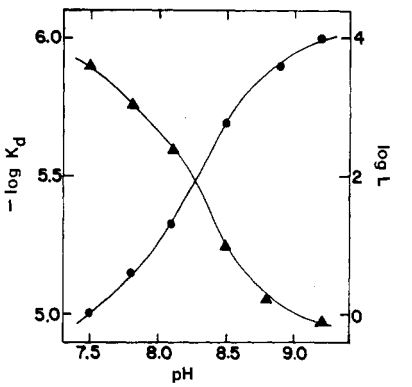


FIGURE 3: Correlation of pH with inducer binding parameters. Apparent inducer affinity and fitted L values are plotted vs. pH for unmodified repressor protein. (▲) Apparent association binding constant; (●) L values.

to the tetrameric species at pH 9.2 (Hill coefficient = 1.9). This cooperativity for the wild-type repressor was also reflected in the inducer association rate constant at high pH; the dependence of k_{obsd} on IPTG concentration was linear at pH 7.2 but exhibited curvature at elevated pH (data not shown).

pH Dependence of Inducer Binding to Unmodified Repressor. Varying the pH from 7.5 to 9.2 resulted in a decrease in the apparent inducer affinity of ~7-fold for unmodified repressor; over the same pH range, the Hill coefficient increased from 1.0 to 1.9. The midpoint of the transition was pH 8.3 (Figure 3), a value consistent with previous results (Friedman et al., 1977).

Thermodynamic Analysis. The two extreme paths from unliganded to completely liganded protein are shown in Table II with the total free energies of ligand binding to repressor proteins under various conditions. As predicted by thermodynamic principles, the total free energy for one pathway ($\Delta G_1 = 4\Delta G_{R/I} + 2\Delta G_{RI/O}$) is equivalent to the total free energy of the alternate pathway ($\Delta G_2 = 2\Delta G_{R/O} + 4\Delta G_{RO/I}$) for each of the protein species. Changes in an equilibrium binding constant for one ligand are compensated by at least one of the other binding equilibria (Tables I and II). The ΔG_1 and ΔG_2 values are equivalent only for a stoichiometry of two operators and four inducers per tetramer. These results are consistent with values reported in the literature for inducer and operator fragments (Butler et al., 1977; Ohshima et al., 1974; O'Gorman et al., 1980; Culard & Maurizot, 1981) and with those determined for operator-containing plasmid DNA (Whitson et al., 1986). The total free energy of forming the completely liganded repressor at pH 9.2 is significantly less than at pH 7.5 for unmodified protein. This differential in

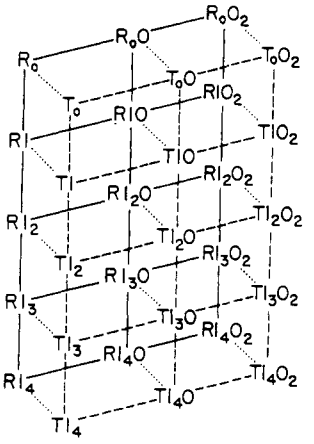


FIGURE 4: Monod–Wyman–Changeux model for binding of inducer and operator DNA. This scheme incorporating two operator binding sites depicts all of the possible transitions. I and O refer to inducer and operator, respectively. R and T states are described in the text. The indicated transitions are allowed.

free energy may be used to stabilize subunit interactions and ionize amino acid side chains or may be dissipated in other ways.

Modeling. The model of Monod–Wyman–Changeux for cooperative systems has been previously utilized to describe the behavior of the lactose repressor protein (O'Gorman et al., 1980). This model requires the following assumptions: (1) the repressor possesses at least one axis of symmetry; (2) each inducer molecule binds to only one site on the monomer; (3) the conformational state of each subunit is limited by association with other subunits; (4) each subunit exists in one of two reversible states designated as R (high inducer affinity; low operator DNA affinity) and T (low inducer affinity; high operator affinity); (5) the symmetry of the protein must be maintained during the conversion from one state to the other. In addition to the above conditions, a model incorporating two operator DNA binding sites was used. O'Gorman et al. (1980) determined that a model with a single operator DNA binding site could not adequately describe their data. Figure 4 presents all of the available transition states in a model with two operator DNA binding sites. In this model, both R and T states bind to operator DNA, although with different affinities. The equilibrium association constants for operator are designated as K_{RO} and K_{TO}; inducer equilibrium association constants are designated as K_{RI} and K_{TI}, respectively. The equilibrium constant, L, is a measure of the relative concentrations of unliganded T and R states in solution ($L = T/R$). A modification which alters the population of T-state subunits relative to R-state subunits will necessarily alter the value of L.

For a tetrameric protein with four inducer and two operator DNA binding sites, eq 1 can be used to predict the fractional

$$Y = \{K_{RI}[I](1 + K_{RI}[I])^3(1 + 2K_{RO}[O] + K_{RO}^2[O]^2) + LcK_{RI}[I](1 + cK_{RI}[I])^3(1 + 2K_{TO}[O] + K_{TO}^2[O]^2)\}/\{(1 + K_{RI}[I])^4(1 + 2K_{RO}[O] + K_{RO}^2[O]^2) + L(1 + cK_{RI}[I])^4(1 + 2K_{TO}[O] + K_{TO}^2[O]^2)\} \quad (1)$$

saturation of repressor at varying inducer concentrations. The ratio between K_{TI} and K_{RI} is designated c. By altering one or more of the available parameters, it is possible to fit experimentally derived curves.

In TMS buffer, the following experimental equilibrium association constants were utilized in generating the fits: $K_{TO} = 1.7 \times 10^9$ M^{-1} (operator affinity at high pH), $K_{RO} = 6.3 \times 10^6$ M^{-1} (operator affinity in the presence of inducer), $K_{RI} = 7.1 \times 10^5$ M^{-1} (inducer affinity at low pH), $K_{TI} = 7.7 \times 10^3$ (inducer affinity in the presence of operator at high pH), and $c = 0.011$. The simplest model is to vary L (the ratio of the two conformational states) and keep all other binding constants fixed. We have therefore assumed that the intrinsic constants are unchanged by modification and increased pH; therefore, any changes in the observed equilibrium association constants are assigned to shifts in T- and R-state equilibria. The following data support this assumption: (1) DNA binding affinities were unaffected by increased pH. (2) The inducer affinity of the monomeric mutant was pH independent, as was the inducer affinity for MMTS-modified repressor. On the basis of thermodynamic limitations, the intrinsic association constant for inducer binding to operator-bound repressor must also be unchanged. (3) Inducer and nonspecific DNA binding affinities were unaffected by modification of the protein with MMTS. Although reaction of Cys-281 affects operator affinity, this residue is located in a region involved in subunit interaction (Daly & Matthews, 1986; Daly et al., 1986) and therefore presumably cannot directly contact operator DNA.

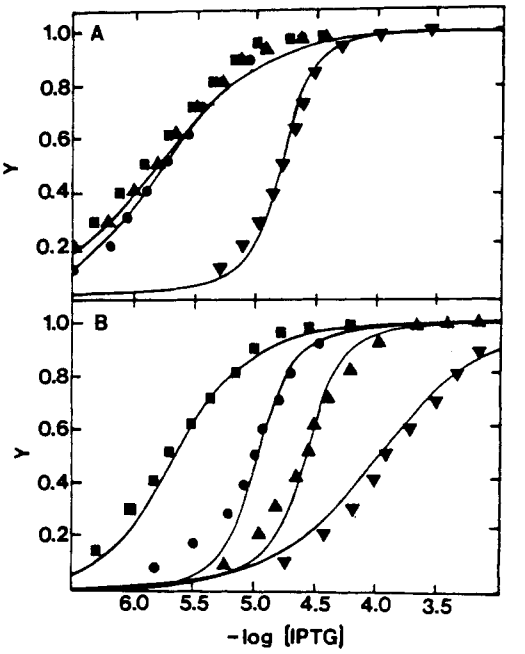


FIGURE 5: Theoretical curves for inducer binding to the repressor in the absence and presence of operator DNA using the Monod–Wyman–Changeux model. Symbols are experimental data from Figures 1 and 2; lines are fits generated by eq 1 using the L value indicated and the following fixed parameters: $K_{RI} = 7.1 \times 10^5$ M^{-1}, $K_{RO} = 6.3 \times 10^6$ M^{-1}, $K_{TO} = 1.7 \times 10^9$ M^{-1}, and $c = 0.011$. (■) MMTS–repressor, pH 7.5 ($L = 0.0001$); (●) MMTS–repressor, pH 9.2 ($L = 0.03$); (▲) repressor, pH 7.5 ($L = 1$); (▼) repressor, pH 9.2 ($L = 10000$). (A) Inducer binding in the absence of operator DNA. (B) Inducer binding in the presence of 2×10^{-6} M operator DNA.

Theoretical inducer binding curves were generated for repressor in the presence and absence of operator. Figure 5 shows theoretical inducer binding curves superimposed on experimentally determined points. For all of the simulated inducer binding curves, only the value of L was varied. For unmodified repressor at pH 7.5, a value of $L = 1$ (an equal population of R and T states) yielded a fit for the experimental inducer binding curves. At pH 9.2, however, the fitted value of L was 10^4. Consistent with the model prediction, no cooperativity was observed in the presence of operator DNA at high pH. The observed cooperativity for inducer binding at high pH in the absence of operator can be ascribed to a shift of the repressor into a conformation favoring operator binding. Dunaway et al. (1980) demonstrated that the association rate for inducer binding to repressor–operator complex was di-

minished ~5-fold compared to unliganded repressor. The similarity in parameters for inducer binding at high pH and in the presence of operator suggests similar effects of these states on the conformation of the protein.

Inducer binding curves for MMTS-modified repressor at pH 7.5 in the absence and presence of operator DNA were fit by decreasing L to 0.0001. In the absence of operator DNA, the theoretical inducer binding curve exhibits no cooperativity with an inducer half-saturation concentration of 1.5×10^{-6} M. In the presence of saturating operator DNA, almost no cooperativity was observed. The predicted inducer concentration at half-saturation is consistent with the observed loss of differential between operator–repressor and operator–inducer–repressor binding constants. Modification of cysteine-281 therefore appears to shift the repressor tetramer into the R state. The absence of pH dependence for inducer binding to T-41 monomeric repressor indicates that quaternary structure may be necessary for formation of the T-state and specific operator binding activity. Inducer binding to MMTS-modified repressor at pH 9.2 was best modeled by using an L of 0.03. Although this protein species is shifted into the R state, the effect of MMTS modification at cysteine-281 is modulated by high pH. Thus, there appears to be an additional region of the protein which influences binding as a function of pH. The position of mutations which affect operator and inducer binding in the "hinge region" suggests this as a possible site (Chakerian et al., 1985; Müller-Hill et al., 1977; Miller, 1979; Miller et al., 1977; Pfahl et al., 1974).

Discussion

MMTS modification and changes in pH appear to elicit alterations in lactose repressor protein structure. At high pH, inducer binding to the repressor protein was cooperative. Ohshima et al. (1974) previously observed a Hill coefficient of 1.2 at pH 8.2 and predicted the cooperative inducer binding to operator-bound repressor subsequently demonstrated by O'Gorman et al. (1980). For each of the protein species analyzed in this study, theoretical binding curves which fit the experimental data could be generated by varying a single parameter, L. Although binding curves which fit all of the individual experimental inducer binding curves could be generated by varying other parameters, L was the only single parameter whose variation allowed simulation of the entire family of inducer binding curves. The value of L, the equilibrium constant for T and R states, ranged from 10^{-4} for MMTS-modified repressor to 10^4 for repressor at pH 9.2. The difference in ΔG values between pH 7.5 and 9.2 for complete ligation of the unmodified protein corresponds well to $-RT$ ln L; this argument suggests that the differential is due to subunit interactions and/or ionization processes which are involved in the R to T transition.

Equilibrium association constants for inducer binding and fitted L values exhibited pH dependence with a midpoint at pH 8.3 (Figure 3). The midpoint of the presumed shift in conformation of the protein into the T state correlates well with the pK value for deprotonation of cysteine residues. The addition of methanethiol group to cysteine-281 apparently elicits a shift of the protein into the R state. In contrast to the unreacted protein, the inducer binding affinity of MMTS-modified repressor was unaffected by changes in pH. Addition of the methanethiol moiety at cysteine-281 would preclude ionization of this sulfhydryl at increased pH. Modification of cysteine-281 with MNP and DTNB has also been shown to alter inducer binding (Daly et al., 1986), presumably by a shift in the subunit population of the repressor toward the R state. The sulfhydryl proton at Cys-281 does not itself appear to participate in operator binding, but this region is apparently involved in subunit interactions which modulate the ligand binding properties of the protein. Replacement of the adjacent Tyr-282 by serine in the mutant monomeric repressor T-41 results in inhibition of subunit assembly.

The equilibrium between T and R states of the repressor appears to depend upon whether cysteine-281 is capable of protonation/deprotonation. Wu et al. (1976), using temperature jump experiments, concluded that both inducer and a proton bound preferentially to the R state of the lactose repressor. Wu et al. (1976) also predicted that cooperativity for inducer binding would exist at increased pH but were unable to determine which amino acid was involved. From the data presented in this and the preceding papers (Daly & Matthews, 1986; Daly et al., 1986), it appears that cysteine-281 is involved in subunit communication and its state influences repressor conformational states.

The integrity of the region of the repressor monomer which includes Cys-281 and Tyr-282 appears to be necessary for optimal subunit interaction and communication, and the ionization state of cysteine-281 influences the ligand binding properties of the protein through effects on the subunit interactions in the tetramer. Although cysteine residues traditionally have been shown to function either catalytically or structurally by the formation of disulfide bridges, cysteine-281 neither has a catalytic activity nor is involved in the formation of disulfide bridges. This residue apparently plays an interesting and unusual role in modulating the structural dynamics which affect induction/repression of the lactose repressor protein.

Acknowledgments

We thank Dr. J. S. Olson for many helpful discussions.

Registry No. IPTG, 367-93-1; L-cysteine, 52-90-4.

References

Butler, A. P., Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4757–4768.

Chakerian, A. E., Olson, J. S., & Matthews, K. S. (1985) *J. Mol. Biol. 183*, 43–51.

Culard, F., & Maurizot, J. C. (1981) *Nucleic Acids Res. 9*, 5175–5184.

Daly, T. J., & Matthews, K. S. (1986) *Biochemistry* (second of three papers in this issue).

Daly, T. J., Olson, J. S., & Matthews, K. S. (1986) *Biochemistry* (first of three papers in this issue).

Dunaway, M., Olson, J. S., Rosenberg, J. M., Kallai, O. B., Dickerson, R. E., & Matthews, K. S. (1980) *J. Biol. Chem. 255*, 10115–10119.

Friedman, B. E., Olson, J. S., & Matthews, K. S. (1977) *J. Mol. Biol. 111*, 27–39.

Ganem, D., Miller, J. H., Files, J. G., Platt, T., & Weber, K. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3165–3169.

LePecq, J.-B., & Paoletti, C. (1966) *Anal. Biochem. 17*, 100–107.

Lillis, M., Nick, H., Lu, P., Sadler, J., & Caruthers, M. (1982) *Anal. Biochem. 120*, 52–58.

Miller, J. H. (1979) *J. Mol. Biol. 131*, 249–258.

Miller, J. H., & Reznikoff, W. S., Eds. (1980) *The Operon*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miller, J. H., Ganem, D., Lu, P., & Schmitz, A. (1977) *J. Mol. Biol. 109*, 275–301.

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

Müller-Hill, B., Gronenborn, B., Kania, J., Schlotmann, M.,

& Beyreuther, K. (1977) in *Nucleic Acid-Protein Recognition* (Vogel, H., Ed.) pp 219-236, Academic Press, New York.
O'Gorman, R. B., Rosenberg, J. M., Kallai, O. B., Dickerson, R. E., Itakura, K., Riggs, A. D., & Matthews, K. S. (1980) *J. Biol. Chem. 255*, 10107-10114.
Ohshima, Y., Mizokoshi, T., & Horiuchi, T. (1974) *J. Mol. Biol. 89*, 127-136.
Pfahl, M., Stockter, C., & Gronenborn, B. (1974) *Genetics 76*, 669-679.
Riggs, A. D., Bourgeois, S., Newby, R., & Cohn, M. (1968) *J. Mol. Biol. 34*, 365-368.
Rosenberg, J. M., Kallai, O. B., Kopka, M. L., Dickerson, R. D., & Riggs, A. D. (1977) *Nucleic Acids Res. 4*, 567-572.
Schmitz, A., Schmeissner, U., Miller, J. H., & Lu, P. (1976) *J. Biol. Chem. 251*, 3359-3366.
Whitson, P. A., Olson, J. S., & Matthews, K. S. (1986) *Biochemistry 25*, 3852-3858.
Wu, F. Y.-H., Bandyopadhyay, P., & Wu, C.-W. (1976) *J. Mol. Biol. 100*, 459-472.

Proteolytic Activation of the Canine Cardiac Sarcoplasmic Reticulum Calcium Pump†

Madeleine A. Kirchberger,* Douglas Borchman, and Chinnaswamy Kasinathan
Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

Received January 3, 1986; Revised Manuscript Received April 29, 1986

ABSTRACT: Mild trypsin treatment of canine cardiac microsomes consisting largely of sarcoplasmic reticulum vesicles produced a severalfold activation of oxalate-facilitated calcium uptake. The increase in calcium uptake was associated with an increase in ATP hydrolysis. Proteases other than trypsin were also effective although to a lesser degree. Trypsin produced a shift of the Ca^{2+} concentration dependency curve for calcium uptake toward lower Ca^{2+} concentrations, which was almost identical with that produced by phosphorylation of microsomes by cyclic AMP dependent protein kinase when the trypsin and the protein kinase were present at maximally activating concentrations. The Hill numbers (±SD) of the Ca^{2+} dependency after treatment of microsomes with trypsin (1.5 ± 0.1) or protein kinase (1.7 ± 0.1) were similar and were not significantly different from those for untreated control microsomes (1.6 ± 0.1 and 1.8 ± 0.1, respectively). Autoradiograms of sodium dodecyl sulfate-polyacrylamide electrophoretic gels indicate that ^{32}P incorporation into phospholamban (M_r 27.3K) or its presumed monomeric subunit (M_r 5.5K) was markedly reduced when trypsin-treated microsomes were incubated in the presence of cyclic AMP dependent protein kinase and [γ-^{32}P]ATP compared to control microsomes incubated similarly but pretreated with trypsin inhibitor inactivated trypsin. The activation of calcium uptake by increasing concentrations of trypsin was paralleled by the reduction of phosphorylation of phospholamban. Trypsin treatment of microsomes previously thiophosphorylated in the presence of cyclic AMP dependent protein kinase and [γ-^{35}S]thio-ATP did not result in a loss of ^{35}S label from phospholamban, which suggests that phosphorylation of phospholamban protects against trypsin attack. Trypsin treatment of microsomes prepared from rabbit fast skeletal muscle, which does not contain phospholamban, did not stimulate calcium uptake. However, autoradiograms of gels of rabbit skeletal muscle and canine cardiac microsomes incubated under conditions favorable for the formation of the 100-kilodalton acylphosphoprotein intermediate of the (Ca^{2+} + Mg^{2+})-activated ATPase reaction showed an identical pattern of effects of different concentrations of trypsin. These data suggest that trypsin cleaves similar sites on both calcium pump proteins. Therefore, the stimulatory effect of trypsin on cardiac microsomal calcium uptake does not appear to be due to a direct effect on the calcium pump protein. The data are consistent with a model in which a segment of phospholamban is in communication with the cytoplasm. If the segment is cleaved proteolytically, the basal rate of calcium transport is increased. The proteolytic activation of calcium transport would suggest that the presence of this unphosphorylated cytosolic segment has an inhibitory effect on the calcium pump.

Cardiac muscle relaxation occurs as a result of a decrease in cytoplasmic Ca^{2+} and consequent dissociation of calcium from troponin C. The decrease in cytoplasmic Ca^{2+} is brought about by three different mechanisms. The sodium-calcium exchange system (Reuter & Seitz, 1968) and the sarcolemmal calcium pump (Caroni & Carafoli, 1981) transfer Ca^{2+} out of the cell, and the sarcoplasmic reticulum (SR)[1] calcium pump sequesters calcium within the tubular SR network (Michalak, 1985). The relative contributions of these three

† This work was supported by Grant HL 15764 from the U.S. Public Health Service, National Institutes of Health.
* Correspondence should be addressed to this author.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BAEE, sodium benzoyl-L-arginine ethyl ester; EP, phosphoenzyme intermediate of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-activated ATPase protein; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; TEMED, *N*,*N*,*N*',*N*'-tetramethylethylenediamine; cAMP, adenosine cyclic 3',5'-phosphate; kDa, kilodalton(s).

0006-2960/86/0425-5484$01.50/0