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Dissociation of the Lactose Repressor Protein Tetramer Using High Hydrostatic Pressure†

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ABSTRACT: Dissociation of *lac* repressor tetramer by high hydrostatic pressures was monitored with intrinsic tryptophan fluorescence. With the assumption of complete dissociation to monomer, tryptophan polarization data gave $\Delta V_a \sim 170$ mL/mol and the concentration for 50% tetramer dissociation, $C_{1/2}$, was 3.8×10^{-8} M. Upon addition of inducer, the calculated ΔV_a increased to ~220 mL/mol and the $C_{1/2}$ decreased to approximately 1×10^{-8} M, a free energy difference of ~0.7 kcal. These results indicate a modest stabilization of the tetramer by the presence of inducer. Monitoring the average energy of tryptophan emission demonstrated that (1) tetramer dissociation takes place over the same range of pressures as evidenced by the polarization data and (2) IPTG dissociation can be more or less superimposed upon tetramer dissociation depending upon the ligand concentration used. Although the two transitions cannot be separated entirely, the ΔV_a for the region of the pressure dependence dominated by ligand dissociation was 69 mL/mol, an unexpectedly large value. For tetramer modified with methyl methanethiosulfonate, subunit dissociation was shifted to much higher pressures and IPTG dissociation did not occur. The ΔV_a for subunit association was calculated as ~160 mL/mol, and the $C_{1/2}$ was 3.5×10^{-9} M. Interactions at the subunit interface of the modified protein are apparently stronger than in the unmodified protein. The absence of inducer dissociation from the MMTS-modified tetramer by the application of high hydrostatic pressure suggests that the volume change for inducer binding to the modified protein is much smaller than that observed for the unmodified repressor.

Specific interactions between proteins and nucleic acids have become the subject of increasing interest in recent years due to their important role in the regulation of biological functions. In addition to those proteins that are directly involved in the processes of transcription, replication, and translation, several proteins involved in the regulation of these events have been studied. Examples of such proteins include the *lac*, *trp*, *gal*, Cro, and C_I repressors and the catabolite activator protein (Miller & Reznikoff, 1980; Freifelder, 1983). Because these proteins exist as oligomers in solution, the equilibria involved in their specific DNA interactions include not only those of the nucleic acid and the effector molecules binding to the oligomer but also the interactions between the individual protein subunits. Although protein–DNA interactions have been examined in some detail (Miller & Reznikoff, 1980; Cold Spring Harbor Symposium, 1982; Ohlendorf & Matthews, 1983; Watson, 1983), the protein–protein interactions have not been adequately characterized due to the relatively high affinity between the subunits.

The lactose repressor protein controls expression of the metabolic enzymes for the *lac* operon by specific interaction at the operator site in the genome (Miller & Reznikoff, 1980). Binding to this site is modulated by interaction of sugar ligands with the tetrameric repressor protein (M_r 150 000); inducers decrease the affinity of the protein for operator DNA, while anti-inducers stabilize the repressor–operator complex (Miller & Reznikoff, 1980). The alterations in operator affinity elicited by these ligands are mediated by conformational changes in the repressor protein structure (Miller & Reznikoff, 1980). The binding of inducer to the free repressor protein is noncooperative at neutral pH but is cooperative (Hill coefficient = 1.4) in the presence of 40-bp[1] operator DNA fragments (O'Gorman et al., 1980). This cooperativity presumably reflects subunit–subunit interactions that influence the inducer binding behavior of the protein when complexed with its target

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[1] Abbreviations: ABP, arabinose binding protein; bp, base pair; DTE, dithioerythritol; IPTG, isopropyl β-D-thiogalactoside; MMTS, methyl methanethiosulfonate; SDS, sodium dodecyl sulfate.

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site on the DNA and may reflect alterations in subunit affinity.

Information regarding the interface between the subunits in the tetrameric repressor has been obtained from a variety of methods. Mutants have been produced that are primarily monomeric, bind inducer with the wild-type affinity, but do not bind to operator DNA, and generally do not bind to phosphocellulose (Schmitz et al., 1976; Miller, 1980). These mutations map to the carboxy-terminal region of the protein. Monoclonal antibodies have been isolated that do not react with native tetrameric wild-type lactose repressor but do react with partially purified monomeric mutant repressor and with wild-type repressor dissociated with SDS (Sams et al., 1985). The carboxy-terminal region of the protein contains the epitopes for recognition by this antibody (Sams et al., 1985). Recent chemical studies using methyl methanethiosulfonate (MMTS) have demonstrated a significant effect of reaction at Cys-281 on the kinetics of inducer binding (Daly et al., 1986). In addition, the modification of this cysteine residue abrogates the changes in equilibrium and kinetic parameters observed with pH variation (pH 7.0–9.5) for the unmodified protein (Friedman et al., 1977; Daly & Matthews, 1986b). Purification and characterization of a mutant monomeric repressor (Tyr-282 → Ser) have indicated that inducer equilibrium and kinetic binding constants are similar to those for wild-type repressor protein at neutral pH; further, reaction with MMTS or increasing pH elicits minimal change in the monomer–inducer binding constants (Daly & Matthews, 1986a). These results are consistent with substantial influence of intersubunit contacts on the interaction of the lactose repressor protein with sugar ligands. One site of subunit interaction appears to be near the region including Cys-281 and Tyr-282, as alterations at these sites lead to significant effects on the oligomeric structure or cooperative properties of the protein.

In this work we have monitored the high-pressure dissociation of the *lac* repressor tetramer and the repressor–sugar ligand dissociation with fluorescence spectroscopy; we have utilized conditions known to alter the properties of the protein to examine the effects on subunit interaction.

Materials and Methods

Theoretical Considerations. The application of high hydrostatic pressure elicits the dissociation of oligomeric proteins (Payens & Heremans, 1969; Collen et al., 1970; Heremans, 1974; Schade et al., 1980; Jeanicke et al., 1981; Müller et al., 1981; Paladini & Weber, 1981a; Weber & Drickamer, 1983). The reasons for this dissociation are threefold: primarily, the solvent molecules can occupy small free spaces at the newly exposed subunit interfaces of the dissociated protein, secondarily, nonpolar interactions are replaced by dipole–dipole and dipole-induced dipole interactions that have shorter interaction distances, and finally, salt bridges are broken and electrostriction follows. Thus, the application of pressure pushes the equilibrium to the side that occupies the least volume, the dissociated form.

At constant temperature the variation in the change in free energy with respect to pressure will correspond to the change in volume, ΔV_T, associated with the reaction.

$$d\Delta G_T/dP = \Delta V_T \quad (1)$$

For a tetramer–monomer equilibrium, the composite dissociation constant ($K_d = K_{T\rightarrow D}K_{D\rightarrow M}^2$) is

$$K_d = 256T_0^3(\alpha^4/(1-\alpha)) \quad (2)$$

where α is the degree of tetramer dissociation and T_0, the total protein concentration expressed as tetramer. Thus

$$\ln(\alpha_p^4/(1-\alpha_p)) = \ln(\alpha_0^4/(1-\alpha_0)) + p\Delta V_a/RT \quad (3)$$

where α_p is the degree of dissociation at pressure p, α_0 that at atmospheric pressure; ΔV_a the volume change upon tetramer association, R the gas constant, and T the Kelvin temperature. The slope of the plot of $\ln(\alpha^4/(1-\alpha))$ vs. pressure yields the volume change associated with the reaction and the intercept yields the dissociation constant at atmospheric pressure. If the slope is constant, interface compressibility can be considered negligible. The concentration at which the tetramer is 50% dissociated can be calculated from the K_d with the relation:

$$C_{1/2} = (K_d/32)^{1/3} \quad (4)$$

When an oligomeric protein is subjected to high hydrostatic pressure in presence of its ligand, the pressure at which half-dissociation is observed, $p_{1/2}$, may be shifted. A higher $p_{1/2}$ will be observed if ligand binding stabilizes the oligomer, and a lower $p_{1/2}$ will be observed if ligand binding destabilizes the oligomer. The dissociation profile will be a function of the concentration of ligand used as well as the volume changes and affinities of the ligand for oligomeric and dissociated forms.

If the concentration of ligand is much larger than that of the protein, then the equilibrium constant of ligand binding to a subunit (associated or dissociated) can be expressed as

$$K_x = [X][S]/[SX] = X_0\frac{\alpha}{1-\alpha} \quad (5)$$

where [X] is the concentration of free ligand, X_0 the total concentration of ligand, [S] the concentration of unbound subunit, and [SX] the concentration of liganded subunit. The expression for K_x in eq 5 may be substituted into eq 3 for calculations of ΔV_a and K_d at atmospheric pressure for the binding of the ligand to the protein.

The effect of pressure on the *lac* repressor tetramer was monitored by observing changes in the fluorescence emission spectrum and the polarization of fluorescence of the intrinsic tryptophan residues. Paladini and Weber (1981a) observed a decrease in the polarization and a shift toward longer wavelength of the fluorescence emission spectrum of the high-pressure dissociated enolase dimer. A similar shift was observed by Brewer and Weber (1966) upon dissociation by salt of the same protein. The red-shift in emission is due to the replacement of the native environment by a more polar one, presumably the solvent. For the intrinsic fluorophore studied in this work, tryptophan, the excited-state dipole moment, μ^*, is larger than the ground-state dipole moment, μ, and the increase in polarity upon substitution of subunit contacts by contacts of higher polarity leads to a shift of the fluorescence emission toward lower energies (red-shift) (Bayliss & McRae, 1954; Lippert, 1957; Bakhshiev, 1964; Anufrieva et al., 1970; Macgregor & Weber, 1981). The average energy of emission is characterized by the center of spectral mass, $\bar{\nu}_g$, of the fluorescence spectra, calculated with the relation

$$\bar{\nu}_g = \sum_i F_i\bar{\nu}_i/\sum_i F_i \quad (6)$$

F_i is the fluorescence intensity at a given wavenumber, $\bar{\nu}_i$. Shifts as small as 10 cm^{-1} can be detected. The degree of dissociation, α, is then calculated from either anisotropy or center of spectral mass data assuming that at any pressure there are only two characteristic components, m, monomer, and o, oligomer. This assumption is probably not applicable to all cases and must be considered a useful approximation

$$\alpha = (1 + Q(A_p - A_m)/(A_o - A_p))^{-1} \quad (7)$$

(Paladini & Weber, 1981a) or

$$\alpha = (1 + Q(\bar{\nu}_{gp} - \bar{\nu}_{gm})/(\bar{\nu}_{go} - \bar{\nu}_{gp}))^{-1} \quad (8)$$

where Q is the relative quantum yield of monomer to oligomer and A_p, A_o, $\bar{\nu}_{gp}$, $\bar{\nu}_{go}$, A_m, and $\bar{\nu}_{gm}$ are the values of the anisotropy and center of mass at pressure p and of the oligomer and monomer, respectively.

Apparatus. The high-pressure apparatus used in the experiments was described in detail by Paladini and Weber (1981b). Polarizations were measured with the photon-counting polarization instrument in the T format and corrected with T-format scrambling factors. Spectra were recorded on a microprocessor-controlled photon-counting scanning fluorimeter (Royer, 1985). All spectra are technical; that is, they are not corrected for the wavelength response of the phototube and monochromator. The relatively small changes effected by this correction should not appreciably alter the differences in the center of spectral mass calculated from the uncorrected spectra, especially in the ultraviolet region of the spectrum where the correction factors are practically independent of wavelength. Equilibration was systematically 10 min at each pressure. All volume changes and dissociation constants were calculated using a least-squares fit of the data.

Proteins. The wild-type *lac* repressor tetramer was purified from *Escherichia coli* CSH46 according to the methods described by Rosenberg et al. (1977) with modifications indicated by O'Gorman et al. (1980). The protein (>95% pure by SDS gel electrophoresis) was stored at −70 °C. For MMTS-modified repressor, a 50-fold molar ratio of MMTS to monomer was added to protein dialyzed into N_2-purged 0.1 M Tris-HCl, pH 7.6; following a 30-min reaction period, reagent was removed by dialysis.

Assay of Activity. Isopropyl β-D-thiogalactoside (IPTG) binding activity was determined by nitrocellulose filter binding or ammonium sulfate precipitation (Bourgeois, 1971) or by direct fluorescence titration (O'Gorman et al., 1980). Operator DNA binding was determined with labeled λp*lac* 5 DNA or 40-bp operator DNA fragments by nitrocellulose filtration methods (Riggs et al., 1968). Previous to experiments, the repressor solution was thawed, diluted into 0.1 M Tris-HCl, pH 7.5 or 9.0, 0.1 M KCl, and 10^{-4} M DTE; when MMTS-modified repressor was used, DTE was omitted from the buffer. The solution was then dialyzed against the same buffer overnight. The concentration was determined by ultraviolet absorption by using a subunit molar extinction coefficient of 2.25×10^4 cm^2 mM.

Chemicals. Glucose was purchased from Sigma Chemical Co., and IPTG was purchased from Calbiochem-Behring. MMTS was obtained from Aldrich Chemical Co. Tris Ultrol from Calbiochem-Behring was used in all high-pressure buffers because of its high purity and low pK_a dependence upon pressure (Neuman et al., 1973). All glassware was washed in nitric acid before use, and all water was Millipore filtered to 18-MΩ resistance. Buffer background was always less than 2% of the signal. The high-pressure cuvettes were silanized with a 5% solution of dimethyldichlorosilane in chloroform in order to prevent the protein from adhering to the walls of the quartz cuvette. The cuvette was rinsed several times with the silane solution, rinsed with 50 mL of water, and finally baked for 2 h at 180 °C.

Results and Discussion

Pressure Dependence of the Intrinsic Fluorescence Polarization. The pressure dependence of the intrinsic polarization of the *lac* repressor tetramer is shown in Figure 1A. While the sigmoidal shape of the curve generally indicates a dissociation transition, the small change and relatively low signal-to-noise ratio precluded a serious study. However, if we assume that dissociation was complete to monomer, the volume change for tetramer association calculated from the slopes of the plots of $\ln(K_{d_p}/256T_0^3)$ vs. pressure is 168 ± 4 mL/mol, and the average $C_{1/2}$ calculated from the extrapolated K_d at atmospheric pressure is 3.8×10^{-8} M (Figure 2, Table I). The constant slope observed in these plots in repeated experiments indicates that the compressibility of the newly exposed subunits is small. Also evident from the plot in Figure 1A is the large degree of hysteresis upon the release of pressure. Apparently, the dissociated monomers tend to drift away from their original conformation and thus do not reassociate to precisely native

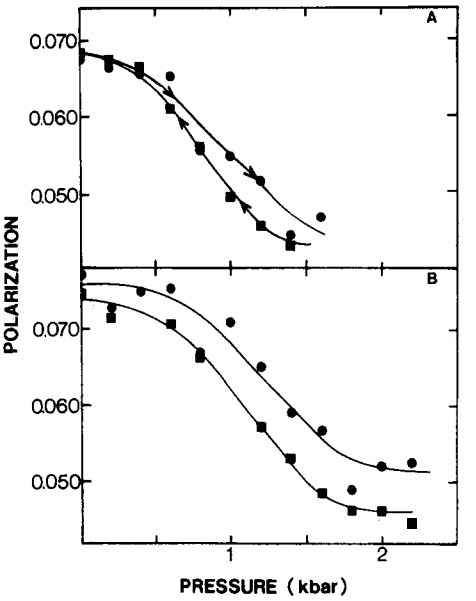

FIGURE 1: Polarization vs. pressure for the *lac* repressor tetramer. Repressor at the indicated concentrations in 0.1 M Tris-HCl, pH 7.5, 0.1 M KCl, and 10^{-4} M DTE was exposed to increased pressure, and the polarization was measured as described in Materials and Methods. (A) 1×10^{-6} M repressor tetramer: (●) forward; (■) reverse. (B) (■) 5×10^{-7} M repressor tetramer, 5×10^{-4} M IPTG; (●) 1×10^{-6} M repressor tetramer, 1.8×10^{-3} M IPTG. Excitation wavelength was 280 nm, and emission was monitored with a WG 320 Corning filter.

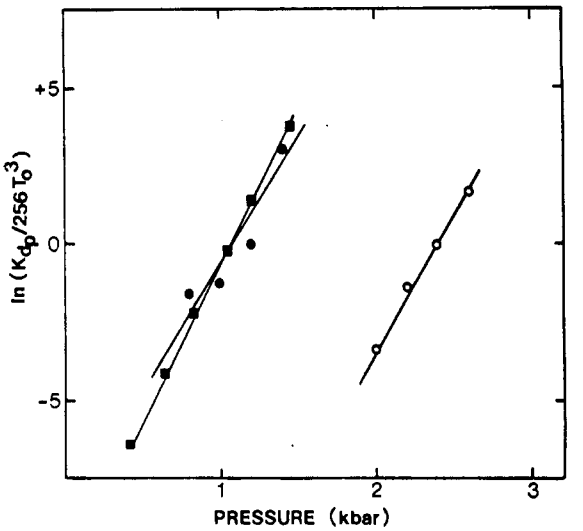

FIGURE 2: Plot of ln $(K_{d_p}/256T_0^3)$ vs. pressure. The data in Figure 1 were analyzed with eq 2 and 3: (●) 1×10^{-6} M repressor tetramer; (■) 5×10^{-7} M repressor tetramer in the presence of 5×10^{-4} M IPTG; (○) 6.6×10^{-7} M MMTS–repressor tetramer in the presence of 4×10^{-3} M IPTG.

Table I: Apparent Volume Changes and Dissociation Constants for Tetramer Dissociation[a]

protein	protein concn (μM)	IPTG concn (mM)	ΔV_a (mL/mol)	K_d (M)	$C_{1/2}$ (M)
repressor	0.5–1.0	0	168	1.8×10^{-21}	3.8×10^{-8}
repressor	1.0	1.8	217	3.0×10^{-22}	2.1×10^{-8}
repressor	0.5	0.5	217	2.0×10^{-23}	8.6×10^{-9}
MMTS–repressor	0.7	4.0	157	1.4×10^{-24}	3.5×10^{-9}

[a] Volume changes and K_d values were calculated from plots of (ln $K_{d_p}/256T_0^3$) vs. pressure. $C_{1/2}$ was calculated from eq 4.

conformation. This type of conformational drift has been seen upon dilution dissociation of the enolase dimer (Xu & Weber, 1982) and upon the high-pressure dissociation of both lactate dehydrogenase tetramer (King & Weber, 1986) and β-subunit dimer of tryptophan synthase (Silva et al., 1986).

In order to determine the effect of ligation upon the dissociation of the *lac* repressor tetramer, the pressure dependence of the polarization was repeated in presence of the inducer ligand, IPTG (Figure 1B). The dissociation profile of the repressor tetramer in the presence of IPTG is similar to that in its absence; however, the transition occurs at slightly higher pressures. It can be seen that if the concentrations of tetramer and IPTG are increased, the shift of $p_{1/2}$ to higher pressures indicates that the tetramer is stabilized by IPTG. The volume change for tetramer association calculated from the slope of the plot of ln ($K_{d_p}/256T_0^3$) vs. pressure (Figure 2) is 217 mL/mol for both solutions. The $C_{1/2}$ values are 2.1×10^{-8} and 8.6×10^{-9} M, respectively, for the high and low concentration solutions. From these measurements, the volume change for tetramer association appears to be greater and the average concentration for subunit dissociation decreases when IPTG is added to the solution. The shift in $p_{1/2}$ as the concentrations of IPTG and repressor are increased is approximately 280 bar. The change in $p_{1/2}$, $\Delta p_{1/2}$, with concentration is given by

$$\Delta p_{1/2} = (RT/\Delta V_a) \ln (C_2/C_1)^n \quad (9)$$

where n has the value of 1 for a tetramer–dimer equilibrium and 3 for a tetramer–monomer equilibrium. ΔV_a in eq 9 is originally calculated by assuming one or the other equilibria and will therefore be different in the two cases: $\Delta V_a(4\rightarrow2)$ and $\Delta V_a(4\rightarrow1)$, respectively. From our experimental data $\Delta V_a(4\rightarrow2)$ = 124 mL/mol and $\Delta V_a(4\rightarrow1)$ = 217 mL/mol. From these values and eq 9, we get corresponding midpoint pressure shifts of 110 and 211 bar. The observed shift of $p_{1/2}$, 280 bar, differs from these by 150% and 33%, respectively. As shown below, the value of 217 mL/mol obtained for the IPTG–repressor complex must contain a considerable contribution from the volume change upon formation of this complex, which we calculate to be −69 mL/mol. In the limit of complete IPTG dissociation the corrected value for $\Delta V_a(4\rightarrow1)$ would be 148 mL, which is very close to the experimental value in the absence of IPTG (168 mL/mol, Table I). With the latter $\Delta V_a(4\rightarrow1)$, eq 9 gives a pressure shift of 263 bar, within 6% of the experimental value. On the basis of these observations, we conclude that in the pressure dissociation of the repressor a tetramer–monomer equilibrium obtains. The absence of hysteresis in reassociation with inducer present indicates that ligand addition favors the recovery to the native form after dissociation or prevents conformational drift of the dissociated monomers.

Pressure Dependence of the Intrinsic Fluorescence in the Presence of IPTG. In the presence of inducer, the intrinsic fluorescence emission spectrum of repressor shifts toward lower wavelength and the bandwidth narrows due to the protection of Trp-220, which appears to be in or near the IPTG binding site (O'Gorman & Matthews, 1977a,b; Sommer et al., 1976; Laiken et al., 1972; Chakerian et al., 1985). Repressor binds 4 mol of inducer per mole of tetramer noncooperatively in absence of operator DNA with a K_d of 4×10^{-6} M (O'Gorman et al., 1980), while the K_d for IPTG binding to wild-type monomer has not been measured. The K_d for inducer binding to mutant monomeric repressor T-41 is similar to wild-type tetramer (Daly & Matthews, 1986a).

Intrinsic fluorescence emission spectra of *lac* repressor in the presence of IPTG at atmospheric pressure and at 2.4 kbar are shown in Figure 3. The profiles of the center of spectral mass, $\bar{\nu}_g$, as a function of pressure for two concentrations of *lac* repressor tetramer in presence of 4 mM IPTG are shown in Figure 4A. In both cases the average energy of emission shifts to the red (lower energies) by approximately 500 cm^{-1} at increased pressures, and the reversibility of all transitions was 95–100%. Because the only difference between the two

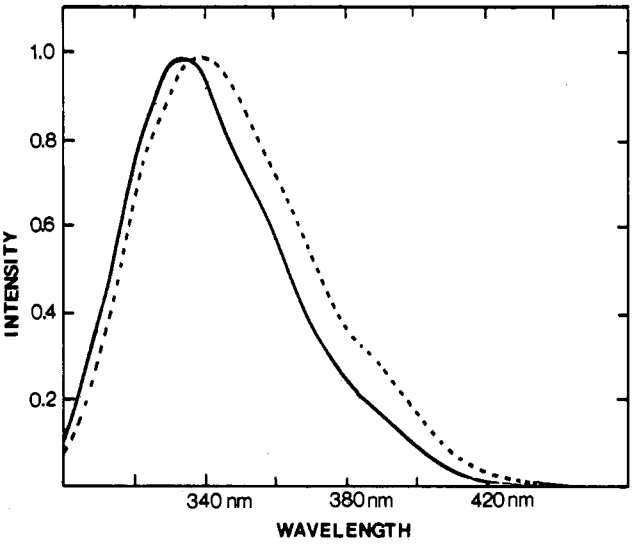

FIGURE 3: Intrinsic fluorescence emission spectra of the *lac* repressor. Repressor (1×10^{-6} M tetramer) fluorescence was measured in presence of IPTG (4×10^{-3} M) at atmospheric pressure (solid line) and at 2.4 kbar (dashed line). Excitation wavelength was 280 nm. Emission was monitored from 300 to 450 nm.

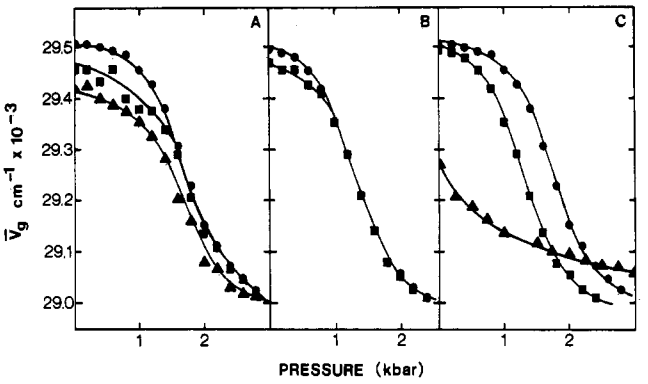

FIGURE 4: Center of spectral mass vs. pressure. Center of spectral mass ($\bar{\nu}_g$) was measured for repressor complexed with ligands in 0.1 M Tris-HCl, 0.1 M KCl, and 10^{-4} M DTE, pH 7.5 (except as indicated). Excitation wavelength was 280 nm. (A) (●) 1×10^{-6} M repressor tetramer, 4×10^{-3} M IPTG; (■) 1×10^{-7} M repressor tetramer, 4×10^{-3} M IPTG; (▲) 1×10^{-6} M repressor tetramer, 4×10^{-3} M IPTG, pH 9.0. (B) (●) 1×10^{-6} M repressor tetramer, 1×10^{-3} M IPTG; (■) 1×10^{-7} M repressor tetramer, 1×10^{-3} M IPTG. (C) (■) 1×10^{-6} M repressor tetramer, 1×10^{-3} M IPTG; (●) 1×10^{-6} M repressor tetramer, 4×10^{-3} M IPTG; (▲) 1×10^{-6} M repressor tetramer, 20% glucose.

Table II: Apparent Volume Changes and Dissociation Constants for IPTG Dissociation[a]

repressor concn (μM)	IPTG concn (mM)	pH	ΔV_a (mL/mol)	K_d (M)
1.0	4.0	7.5	67	1.4×10^{-5}
0.1	4.0	7.5	69	1.4×10^{-5}
1.0	1.0	7.5	69	1.3×10^{-5}
0.1	1.0	7.5	70	1.4×10^{-5}
0.1	4.0	9.0	64	3.0×10^{-5}

[a] Volume changes and K_d values were calculated from plots of ln (K_{d_p}/X_0) vs. pressure.

curves is a 10-fold decrease in the tetramer concentration, it would be expected that if the only transition being monitored were that of IPTG dissociation, then the profiles would be superimposed. However, two regions of the pressure dependence of $\bar{\nu}_g$ are evident in Figure 4A. At lower pressures (<1.6 kbar) there is a marked dependence upon the concentration of protein, with some apparent dissociation at atmospheric pressure upon dilution. However, above 1.6 kbar, the curves superimpose. Below 1.6 kbar, the primary transition being followed appears to be dissociation of the tetramer, while above that pressure IPTG dissociation predominates. The apparent IPTG dissociation at such high concentrations (1000-fold the K_d at atmospheric pressure) is somewhat surprising. At 4 mM IPTG the volume change needed to observe 50% dissociation near 2 kbar would be ~75 mL/mol. In fact, from the plots in Figure 5 of ln (K_{d_p}/X_0) vs. pressure, one obtains a volume change for ligand association of 69 mL/mol and a K_d of 1.4×10^{-5} M (Table II). The difference between this latter value and that reported in the literature, 4.0×10^{-6} M, (e.g., O'Gorman et al., 1980), is presumably due to the contribution of the low-pressure protein dissociation to the overall pressure dependence of the spectral energy as this contribution cannot be accurately subtracted from the profile.

The observed volume change is very large for ligand binding. The effects of pressures below 3 kbar upon ligand–protein interactions, where protein conformation is not seriously altered, have been thought to be similar to the effects of pressure upon complexes of small molecules and thus to involve volume changes of only a few milliliters per mole (Weber & Drickamer, 1983; Li et al., 1976a,b). While Visser et al. (1977) observed a ΔV_a of approximately 70 mL/mol upon dissociation of FMN from various flavodoxins, the transition occurred at pressures greater than 4 kbar and the large ΔV_a was interpreted as signifying a large change in the protein structure that allowed for the release of the FMN. Zipp and Kauzmann (1973) also observed heme dissociation from apometmyoglobin, but again only at pressures well above 4 kbar, where substantial unfolding of the polypeptide occurs. In the case of the *lac* repressor, where the transition occurs below 3 kbar, the large ΔV_a found for IPTG binding may be understood in light of homology between this protein and arabinose binding protein (ABP) (Müller-Hill, 1983; Sams et al., 1984). In ABP the sugar binds to a large cleft between subdomains; by analogy, a similar binding site in *lac* repressor could provide a large space for packing of water molecules. Insertion of water in this binding space upon ligand dissociation would account for the unusually large volume change observed. The volume change is also consistent with the substantial conformational change previously observed upon binding of IPTG (Boschelli et al., 1981; Yang & Matthews, 1976; O'Gorman & Matthews, 1977a,b).

In order to verify that two transitions occur upon the application of high hydrostatic pressures, the dissociation of the tetramer and of the ligand from the tetramer, the pressure

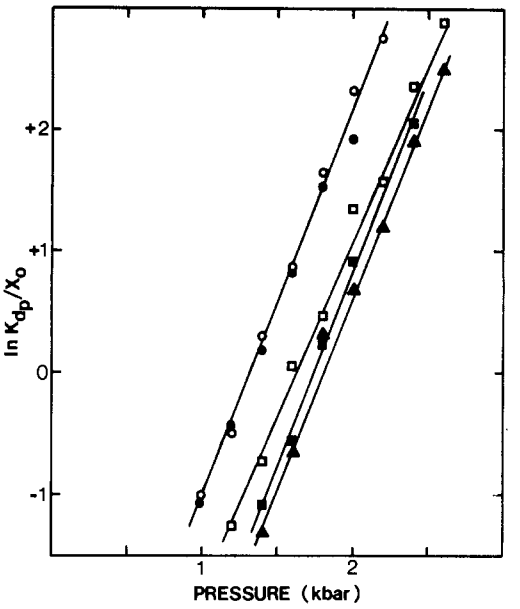

FIGURE 5: ln (K_{d_p}/X_0) vs. pressure for IPTG dissociation. The data in Figure 4 were analyzed with eq 5 and ln K_{d_p} – ln $K_{d_0} = p\Delta V_a/RT$: (▲) 1×10^{-6} M repressor tetramer, 4×10^{-3} M IPTG; (■) 1×10^{-7} M repressor tetramer, 4×10^{-3} M IPTG; (□) 1×10^{-6} M repressor tetramer, 4×10^{-3} M IPTG, pH 9.0; (●) 1×10^{-6} M repressor tetramer, 1×10^{-3} M IPTG; (○) 1×10^{-7} M repressor tetramer, 1×10^{-3} M IPTG.

experiments were repeated in the presence of a lower concentration of IPTG (Figure 4B). The IPTG dissociation is shifted to lower pressures, and thus despite two phases to the profile, IPTG dissociation dominates the region in which the protein dissociation is observed. Although a small dependence upon protein concentration is still evident, the slope in this section is steeper and there is no real plateau at the beginning due to the onset of IPTG dissociation. In addition, lowering the concentration of IPTG may favor protein dissociation. The plots of ln (K_{d_p}/X_0) vs. pressure are shown in Figure 5. For purposes of comparison, the pressure dependence of the center of mass for a single repressor concentration in presence of two IPTG concentrations has been plotted in Figure 4C. The shift in $p_{1/2}$ to lower pressures is 440 bar. This shift depends upon the ratio of the two ligand concentrations employed and upon the volume change involved.

$$\Delta p_{1/2} = RT/\Delta V_a(\ln X_{0_2}/X_{0_1}) \qquad (10)$$

The theoretical $\Delta p_{1/2}$ for a volume change of 69 mL/mol is 437 bar, which agrees well with the experimental results. Table III summarizes the $p_{1/2}$ expected at 5 °C for a ΔV_a of 70 mL/mol for increasing ratios of ligand concentration over the atmospheric K_d for ligand dissociation. The increase in $p_{1/2}$ as the ratio X_0/K_{d_0} increases provides a means to discern whether the observed transition is attributable to ligand dissociation.

Figure 4C shows the pressure dependence of $\bar{\nu}_g$ of repressor in presence of 20% glucose. The $\bar{\nu}_g$ at atmospheric pressure is lower for glucose binding than for IPTG binding, presumably because glucose functions as an anti-inducer and does not elicit the significant conformation change observed with inducer sugars. Due to the very low affinity of the repressor for glucose, 7.2×10^{-2} M (Barkley et al., 1975), the dissociation transition of the glucose should be displaced to much lower pressures than that of IPTG. The center of mass data are summarized in Table II.

Pressure Dependence of the Intrinsic Fluorescence of the lac Repressor in the Presence of IPTG under Modified

Table III: Predicted $P_{1/2}$ for Ratios of Ligand Concentration to K_{d_o}[a]

X_0/K_{d_o}	$P_{1/2}$ (kbar)	X_0/K_{d_o}	$P_{1/2}$ (kbar)
2	0.22	1 000	2.16
10	0.72	10 000	2.88
100	1.44	100 000	3.60

[a] The values were calculated from the following equation for ΔV_a = 70 mL/mol at 5 °C, based on the derivation:

$$\ln\left(\frac{\alpha_p}{1-\alpha_p}\right) = \ln\left(\frac{\alpha_o}{1-\alpha_o}\right) + \frac{\Delta V_a}{RT}(p_{1/2})$$

$$\ln\left(\frac{\alpha_o}{1+\alpha_o}\right) = \ln\frac{X_0}{K_{d_o}}$$

$$\text{if } \alpha_p = 0.5, \text{ then } \ln\left(\frac{\alpha_p}{1-\alpha_p}\right) = 0$$

$$\ln\frac{X_0}{K_{d_o}} = \frac{\Delta V_a}{RT}p_{1/2}$$

Conditions. At high pH, the affinity of IPTG for the repressor is lower than at neutral pH (Friedman et al., 1977). This change in IPTG affinity is evident in the high-pressure dissociation profile as a shift of the IPTG dissociation section of the curve (Figure 4A) approximately 250 bar to lower pressures. From the plot in Figure 5, a ΔV_a for ligand binding of 64 mL/mol is calculated, and the K_d at atmospheric pressure is 3×10^{-5} M (Table II). Thus, the repressor exhibits decreased affinity for the inducer at high pH, the volume change observed is slightly diminished. There also appears to be pH dependence for the section of the curve dominated by subunit dissociation, as it is also shifted to lower pressures. Additionally, there appears to be a fairly large degree of tetramer dissociation at atmospheric pressure. These data are consistent with fewer and/or weaker contacts involved in the interactions.

Modification of *lac* repressor with methyl methanethiosulfonate (MMTS) influences the subunit interactions, and although the affinity of IPTG for the modified repressor is identical with that of unmodified protein, the kinetic rate constants are decreased by a factor of ~50 (Daly et al., 1986). The emission of MMTS-modified protein in the absence of any sugar ligand is identical with that of the unmodified repressor ($\lambda_{max} \sim 345$ nm). Upon binding inducer there is a large shift to higher energies, which is approximately the same as for the unmodified repressor (~900 cm^{-1}). The spectra of MMTS-modified repressor in the presence of IPTG at atmospheric pressure and at 3 kbar are shown in Figure 6. It should be noted that the change in the emission energy upon application of pressure in this case is much smaller than for unmodified protein–inducer complex. The pressure dependence of the center of spectral mass is shown in Figure 7. There is a very long plateau at lower pressures, and the change only begins at 1.6 kbar. The total change of approximately 100 cm^{-1} is complete by 2.8 kbar. The large red-shift (500 cm^{-1}) observed upon dissociation of the IPTG is not observed in this case. Therefore, it can be concluded that the IPTG does not dissociate from the MMTS-modified repressor at pressures below 3 kbar, and the transition observed is attributed to oligomer dissociation. The protein dissociation is shifted to much higher pressures than observed with unmodified tetramer. From the slope of the plot of ln $(K_{d_2}/256T_0^3)$ vs. pressure (Figure 2, Table I) is calculated a ΔV_a of 157 mL/mol, and a $C_{1/2}$ of 3.5×10^{-9} M is found by extrapolation. This increased affinity between the subunits is either a direct result of the MMTS modification or an indirect result of the fact that the IPTG does not dissociate from the modified protein at these pressures. The volume change for IPTG binding to the MMTS-modified repressor may be smaller than for the unmodified protein. In this case, greater pressures would be required to obtain ligand dissociation.

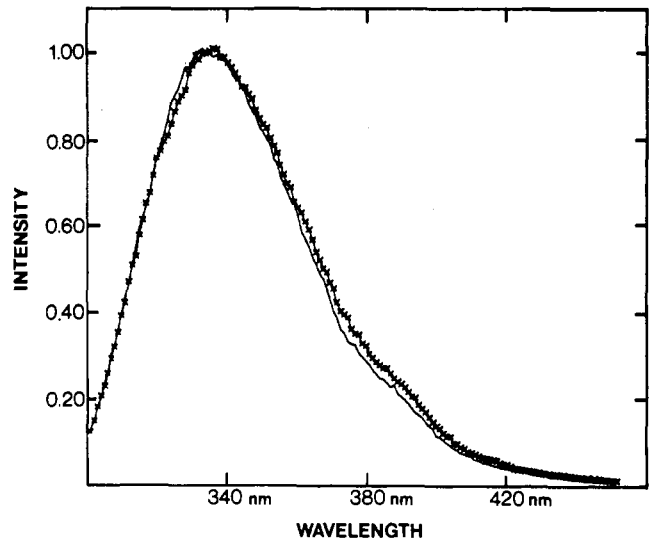

FIGURE 6: Intrinsic fluorescence emission spectra of MMTS-modified repressor. Fluorescence spectra were measured for MMTS-reacted tetramer (6.6×10^{-7} M) in the presence of 4×10^{-3} M IPTG at atmospheric pressure (solid line) and 3.0 kbar (×). Excitation wavelength was 280 nm. Emission was monitored from 300 to 450 nm.

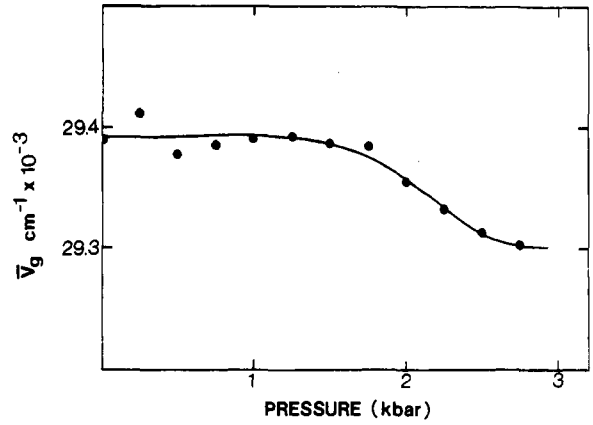

FIGURE 7: Center of spectral mass vs. pressure for MMTS-modified repressor. Center of spectral mass ($\bar{\nu}_g$) was measured for MMTS-repressor tetramer (6.6×10^{-7} M) in the presence of 4×10^{-3} M IPTG. Excitation wavelength was 280 nm. Emission was monitored from 300 to 450 nm.

The binding of IPTG is not salt dependent (Friedman et al., 1977), and therefore the difference in the ΔV_a for IPTG binding to the native and modified repressor cannot be ascribed to differences in the amount of electrostricted water. However, if there are fewer "dead spaces" for solvent to occupy in the MMTS-modified repressor upon dissociation of inducer by pressure, then the ΔV_a would be smaller. A twofold decrease in ΔV_a would be sufficient to put ligand dissociation out of the range used in these studies (<3 kbars). This interpretation would suggest a smaller IPTG binding site in the MMTS-modified repressor. In the terminology of Torgerson et al. (1979) the binding site remains "hard". Alternately, the freedom of the ligand within the binding site may be greater in the MMTS-modified repressor, allowing for compression of the binding site. This would mean that the IPTG–MMTS-modified repressor interactions would be stabilized by the application of pressure: a "hard site" would be changed toward a "soft site".

Conclusions

With the assumption of a transition to monomer, the concentration for 50% tetramer dissociation is found to be 3.8×10^{-8} M, and the ΔV_a for tetramer association is $\sim$170 mL/mol. The addition of IPTG results in a shift to higher pressures of the dissociation profile, an increase in the volume change upon association to $\sim$220 mL/mol, and a $\sim$3-fold decrease in the $C_{1/2}$ for tetramer dissociation. The shift in the $p_{1/2}$ observed when higher concentrations of repressor and IPTG are used is of the magnitude expected for a tetramer to monomer equilibrium. Since the dissociation of IPTG may contribute slightly to the pressure dependence of the polarization, the differences in the dissociation constant and the volume change may contain errors owing to contributions of IPTG dissociation. Monitoring the center of spectral mass yielded dependence of the dissociation profile upon protein concentration, confirming the dissociation deduced from the polarization data. In addition, there appears to be some oligomer dissociation at atmospheric pressure upon dilution of the tetramer from 1×10^{-6} to 1×10^{-7} M, as evidenced by the decrease in the center of spectral mass.

The application of high hydrostatic pressures also results in the dissociation of the ligand, IPTG. The degree of overlap of the protein and ligand dissociation profiles depends upon the concentrations used. The surprisingly large volume change measured for IPTG binding should be correlated with the large change in conformation postulated to occur when inducer binds. The pressure dependence of $\bar{v}_g$ in the presence of saturating glucose demonstrates the decreased affinity of the repressor for this ligand as compared to the IPTG. Increased pressure does not dissociate IPTG from MMTS-modified repressor (below 3 kbar), and subunit interactions appear to be more stable in this modified repressor. The ΔV_a of 157 mL/mol calculated for the tetramer to monomer transition of MMTS-repressor agrees well with those calculated from the polarization data for unmodified repressor, and the $C_{1/2}$, $\sim 4 \times 10^{-9}$ M, reflects the increase in subunit affinity in this tetramer relative to unmodified protein.

Registry No. IPTG, 367-93-1.

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Functional Characterization of Junctional Terminal Cisternae from Mammalian Fast Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: Junctional terminal cisternae are a recently isolated sarcoplasmic reticulum fraction containing two types of membranes, the junctional face membrane with morphologically intact "feet" structures and the calcium pump membrane [Saito, A., Seiler, S., Chu, A., & Fleischer, S. (1984) *J. Cell Biol. 99*, 875–885]. In this study, the Ca^{2+} fluxes of junctional terminal cisternae are characterized and compared with three other well-defined fractions derived from the sarcotubular system of fast-twitch skeletal muscle, including light and heavy sarcoplasmic reticulum, corresponding to longitudinal and terminal cisternae regions of the sarcoplasmic reticulum, and isolated triads. Functionally, junctional terminal cisternae have low net energized Ca^{2+} transport measured in the presence or absence of a Ca^{2+}-trapping anion, as compared to light and heavy sarcoplasmic reticulum and triads. Ca^{2+} transport and Ca^{2+} pumping efficiency can be restored to values similar to those of light sarcoplasmic reticulum with ruthenium red or high $[Mg^{2+}]$. In contrast to junctional terminal cisternae, heavy sarcoplasmic reticulum and triads have higher Ca^{2+} transport and are stimulated less by ruthenium red. Heavy sarcoplasmic reticulum appears to be derived from the nonjunctional portion of the terminal cisternae. Our studies indicate that the decreased Ca^{2+} transport is referable to the enhanced permeability to Ca^{2+}, reflecting the predominant localization of Ca^{2+} release channels in junctional terminal cisternae. This conclusion is based on the following observations: (1) The Ca^{2+},-Mg^{2+}-dependent ATPase activity of junctional terminal cisternae in the presence of a Ca^{2+} ionophore is comparable to that of light sarcoplasmic reticulum when normalized for the calcium pump protein content; i.e., the enhanced Ca^{2+} transport cannot be explained by a faster turnover of the pump. (2) Ruthenium red or elevated $[Mg^{2+}]$ enhances energized Ca^{2+} transport and Ca^{2+} pumping efficiency in junctional terminal cisternae so that values approaching those of light sarcoplasmic reticulum are obtained. (3) Rapid Ca^{2+} efflux in junctional terminal cisternae can be directly measured and is blocked by ruthenium red or high $[Mg^{2+}]$. (4) Ryanodine at pharmacologically significant concentrations blocks the ruthenium red stimulation of Ca^{2+} loading. Ryanodine binding in junctional terminal cisternae, which appears to titrate Ca^{2+} release channels, is 2 orders of magnitude lower than the concentration of the calcium pump protein. (5) By contrast, light sarcoplasmic reticulum has a high Ca^{2+} loading rate and slow Ca^{2+} efflux that are not modulated by ruthenium red, ryanodine, or Mg^{2+}. These studies lead to the conclusion that Ca^{2+} release channels, likely the ones important in excitation–contraction coupling, are localized predominantly in the junctional terminal cisternae. The possible coupling of Ca^{2+} pumping and its relationship to Ca^{2+} release channels are discussed.

The sarcoplasmic reticulum (SR)[1] of muscle is an internal membranous network controlling Ca^{2+} release and uptake, which, in turn, brings about contraction and relaxation [reviews by Endo (1977), Berman (1982), Martonosi (1984), and Inesi (1985)]. The skeletal SR membrane is composed mainly of two morphologically distinct regions, longitudinal tubules and terminal cisternae; the latter are junctionally associated with the transverse tubule via bridging "feet" structures at the triads (Franzini-Armstrong, 1980). The uptake of Ca^{2+} into the lumen of the SR, mediated by the calcium pump protein, enables the muscle to relax. Muscle contraction is triggered by the release of Ca^{2+} from the terminal cisternae via the

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[1] Abbreviations: SR, sarcoplasmic reticulum; RR, ruthenium red; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CaATPase, Ca^{2+},Mg^{2+}-dependent ATPase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; $[Ca^{2+}]_f$ and $[Mg^{2+}]_f$, free (not chelated) Ca^{2+} and Mg^{2+} concentrations, respectively; Tris, tris(hydroxymethyl)aminomethane.

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