Macromolecular Binding Equilibria in the *lac* Repressor System: Studies Using High-Pressure Fluorescence Spectroscopy†

Catherine A. Royer*
Department of Physics, University of Illinois, Urbana, Illinois 61801
Artemis E. Chakerian and Kathleen S. Matthews
Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251
Received September 11, 1989; Revised Manuscript Received January 30, 1990

ABSTRACT: High hydrostatic pressure coupled with fluorescence polarization has been used to investigate protein subunit interactions and protein–operator association in *lac* repressor labeled with a long-lived fluorescent probe. On the basis of observation of a concentration-dependent sigmoidal decrease in the dansyl fluorescence polarization, we conclude that application of high hydrostatic pressure results in dissociation of the *lac* repressor tetramer. The 2-fold decrease in the rotational relaxation time and the high-pressure plateau are consistent with a tetramer to dimer transition. The volume change for tetramer dissociation to dimer is -82 ± 5 mL/mol. The dissociation constant calculated from the data taken at 4.5 °C is 4.3 ± 1.3 nM. The tetramer dissociation constant increases by a factor of 3 when the temperature is raised from 4.5 to 21 °C. A very small effect of inducer binding on the subunit dissociation is observed at 4.5 °C; the K_d increases from 4.5 to 7.1 nM. At 21 °C, however, inducer binding stabilizes the tetramer by approximately 0.8 kcal/mol. Pressure-induced monomer formation is indicated by the curves obtained upon raising the pH to 9.2. The addition of IPTG shifts the pressure transition to only slightly higher pressures at this pH, indicating that the stabilization of the tetramer by inducer is not as marked as that observed at pH 7.1. From the decrease in the polarization of the dansyl repressor–operator complexes, we also conclude that the application of pressure results their dissociation and that the volume change is large in absolute value (approximately 200 mL/mol). The *lac* repressor–operator complex is more readily dissociated upon the application of pressure than the tetramer alone, indicating that operator binding destabilizes the *lac* repressor tetramer.

The *lac* repressor protein controls synthesis of enzymes of the *lac* operon by specific binding to an operator sequence which overlaps the promoter region (Miller & Reznikoff, 1980). The affinity of *lac* repressor for the operator sequence is modulated by binding of β-galactosides which serve as inducers for this operon. Inducer binding causes a conformational change in the *lac* repressor protein which results in a decrease in its affinity for operator (Miller & Reznikoff, 1980) with no effect on nonspecific DNA binding. Competition from the excess nonspecific DNA in the genome then results in dissociation of the repressor–inducer complex, thereby freeing the promoter region to bind RNA polymerase for transcription of the lactose metabolic genes (von Hippel & McGhee, 1972). The *lac* repressor has been shown to be a tetramer of molecular weight 150K (Gilbert & Müller-Hill, 1966; Miller & Reznikoff, 1980) and to contain two operator sites per tetramer (O'Gorman et al., 1980a; Whitson & Matthews, 1986). Like most other repressor–operator systems, the affinities involved in *lac* repressor function are relatively high. The inducer sugar isopropyl β-D-thiogalactoside (IPTG) and the natural inducer allolactose bind with micromolar affinity (O'Gorman et al., 1980b; Barkley et al., 1975), while the protein–operator dissociation constants have been shown to be below nanomolar (O'Gorman et al., 1980a; Riggs et al., 1970a,b; Goeddel et al., 1978). The dissociation of wild-type *lac* repressor tetramer into its dimeric or monomeric forms had been observed previously using high pressure and was estimated to be in the nanomolar range (Royer et al., 1986). The allosteric modulation of protein function implies that binding information must be transferred through the protein matrix. Such site–site interactions most often involve quaternary structural changes implicating changes in the interactions at the subunit interface (Perutz, 1972; Baldwin & Chothia, 1979). It is therefore important to examine protein subunit dissociation in the *lac* repressor/IPTG/operator DNA system in order to elucidate the role of such interactions in the function of the repressor.

A combination of high-pressure and fluorescence techniques was employed in order to observe the dissociation of the *lac* repressor tetramer and the repressor–operator complex. The application of high pressure to solutions of oligomeric proteins has been shown by a number of investigators to lead to the dissociation of oligomers into their respective subunits under favorable conditions of temperature and concentration (Payens & Heremans, 1969; Collen et al., 1970; Heremans, 1974; Jaenicke et al., 1981; Müller et al., 1981; Paladini & Weber, 1981a; King & Weber, 1986; Silva et al., 1986; Ruan & Weber, 1988). This effect is due to both electrostriction of exposed charges and occupation of free spaces by water molecules upon subunit dissociation. Therefore, as pressure is applied to a system in equilibrium, a decrease in the affinity will result if the volume change for the dissociation reaction is negative. The effects of high pressure on biological molecules have been reviewed by Weber and Drickamer (1983) and Heremans (1982).

† This work was supported by National Institutes of Health Grants GM39969 (to C.A.R.) and GM22441 (to K.S.M.) and by Welch Foundation Grant C-576 to K.S.M. Fluorescence measurements and analyses were carried out at the Laboratory for Fluorescence Dynamics at the University of Illinois at Urbana—Champaign. The LFD is jointly supported by the Division of Research Resources of the National Institutes of Health and the University of Illinois.
* To whom correspondence should be addressed.

0006-2960/90/0429-4959$02.50/0

Evidence has been presented (Royer et al., 1986) that the *lac* repressor tetramer could be dissociated using high hydrostatic pressure. However, in this earlier work, the intrinsic tryptophan fluorescence of the repressor protein was used as a probe. Since the fluorescence lifetime of tryptophan is relatively short, a more suitable approach to the study of such a large complex (150 kDa) is covalent labeling of the protein with a long-lived fluorescent probe. In this manner, the fluorescence depolarization observed upon the dissociation of the complex is more sensitive to an increase in the Brownian tumbling rate upon decreasing the size of the complex. We had also shown in our earlier work (Royer et al., 1986) that pressure leads to the destabilization of the repressor–IPTG complex. The effect of high hydrostatic pressure upon protein–DNA interactions was not known at the outset of these studies, although it was shown by Suzuki and Tanigachi (1972) that the DNA double helix itself is stabilized under pressure. We have therefore determined the pressure dependence of the *lac* repressor subunit affinity using protein labeled with 5-(dimethylamino)naphthalene-1-sulfonate (dansyl) at low stoichiometry (Hsieh & Matthews, 1985) and have examined the effects of inducer, temperature, pH, and operator DNA.

Materials and Methods

Protein. The wild-type *lac* repressor protein was purified from *Escherichia coli* according to the methods described by Rosenberg and co-workers (Rosenberg et al., 1977) and modified by O'Gorman et al. (1980a). The protein was stored at −20 °C. IPTG binding was assayed by direct fluorescence titration (O'Gorman et al., 1980b). Operator DNA binding was determined by using ^{32}P-labeled 40 base pair *lac* operator DNA fragments by nitrocellulose filtration methods (Riggs et al., 1968). The protein concentration was determined by ultraviolet absorption using a molar extinction coefficient of 2.25×10^4 M^{-1} cm^{-1} per monomer. Dansyl labeling of the repressor was carried out as follows. Approximately 1 mL of a 1–2 mg/mL solution of the repressor protein was dialyzed against 1 L of a 0.24 M potassium phosphate buffer at pH 8.0 containing 100 mM KCl and 5% dextrose for 3 h. The solution was recuperated, and 10–20 μL of a 0.02 M fresh solution of dansyl chloride in dimethylformamide was added. The reaction was allowed to proceed for approximately 5 min at room temperature. Then 100 μL of a 1 M Tris solution was added to quench the reaction. The reaction mixture was applied to a G-25 superfine desalting column at 5 °C to separate free dye from labeled protein. The dansylated protein solution was then dialyzed for 3 h against 100 mM KCl, 100 mM Tris-HCl, and 0.1 mM DTT, pH 7.1. The distribution of the label on the represson population was characterized by using ion-exchange chromatography. Unmodified and dansyl-*lac* repressor solutions were applied to a CMC-52 cation-exchange column in 100 mM Tris, pH 6.5, and a linear gradient of 0–1 M NaCl was run. Unlabeled *lac* repressor eluted at approximately 0.4 M NaCl. Approximately 80% of the labeled material eluted at 0.03 M NaCl, while the remainder eluted in a small shoulder at 1.5% and another small peak at near 30%. The sample which eluted at 3% displayed a typical dansyl emission with a fluorescence maximum at 500 nm. The average labeling ratio of the sample was 1–2 dansyl residues/tetramer as measured by absorption using a molar extinction coefficient for dansyl of 4500 M^{-1} cm^{-1} at 340 nm. We thus conclude that the labeled solution exhibits a high degree of homogeneity in the distribution of labels over the protein population. No evidence for free dansyl was found in the dialysis buffer either by absorption or by emission spectroscopy. This level of labeling has been shown to affect operator binding by less than 10% (Hsieh & Matthews, 1985). It was noticed that if the protein was labeled and then dialyzed exhaustively to eliminate the free dye, the tetramer could be easily dissociated by dilution techniques, and the degree to which dissociation was observed at atmospheric pressure at a given concentration (between 0.1 and 1 μM in tetramer) was dependent upon the number of days of storage in the cold room. In addition, nonreversibility and nonreproducibility in the pressure dissociation were observed. However, the samples which were freshly prepared and applied to a Sephadex G-25 superfine column to eliminate free dye showed no dissociation upon dilution to 0.1 μM, the detection limit of our instrument in polarization mode, and their pressure-dependent dissociation was both reversible and reproducible.

Apparatus. Polarization measurements were carried out on an ISS Greg PC (ISS, Inc., Urbana, IL) using an excitation wavelength of 340 nm and an emission wavelength of 500 nm. Additionally, a KV-370 cuton filter was placed in the emission path for rejection of scattered exciting light. High-pressure-induced birefringency correction factors were measured by using a scattering solution for all wavelengths between 280 and 600 nm, and those for 340 and 500 nm were used following the method of Paladini and Weber (1981b) to correct the data. The high-pressure apparatus was similar to that described by Paladini and Weber (1980b). Fluorescence lifetimes of the dansyl label were measured on the multifrequency phase and modulation instrument described by Gratton and Limkeman (1983). Global analysis of the lifetime data was performed by using the Globals Unlimited software (Laboratory for Fluorescence Dynamics, Urbana, IL).

Analysis of Binding Data. Polarization values were converted to anisotropy by using the relation

$$A = (2/3)(1/p - 1/3)^{-1} \quad (1)$$

The degree of complex dissociation as a function of pressure was calculated from the anisotropy data by using the equation:

$$\alpha_p = [1 + Q(A_p - A_d/A_a - A_p)]^{-1} \quad (2)$$

where A_p is the anisotropy calculated from the polarization observed at pressure p, A_a is that calculated from the polarization observed for the oligomeric form of the complex at atmospheric pressure, and A_d is that obtained from the polarization of the dissociated form of the complex. The ratio of the intensity at high pressure compared to that at low pressure is the relative quantum yield, Q. Since a tetramer–dimer equilibrium is equivalent mathematically to a dimer–monomer transition, the value of the dissociation constant at atmospheric pressure and also the volume change for the transition were extracted from the intercept and the slope, respectively, of the plots of ln $[\alpha^2/(1 - \alpha)]$ vs pressure (Paladini & Weber, 1981a). Errors on the recovered ΔV_a and K_d values were estimated from the least-squares regression of the ln $[\alpha^2/(1 - \alpha)]$ vs pressure plots calculated by using the standard deviations in the anisotropy data for points at 20% and 80% completion of the dissociation transition. Rotational relaxation times, ρ, were calculated from the limiting anisotropy of the dansyl residue, A_0 (Weber, 1951), the anisotropy calculated from the polarization at pressure p, A_p, and the lifetime, τ, by using the Perrin equation (Perrin, 1926):

$$A_0/A_p - 1 = 3\tau/\rho \quad (3)$$

It was assumed that the percent change in lifetimes was equivalent to those measured for the total intensity. At 21 °C, the change in intensity was very small (less than 10%). At 4.5 °C, the total change over the studied pressure range

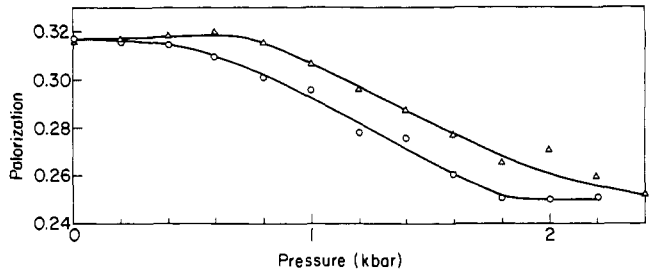

FIGURE 1: Dansyl-*lac* repressor polarization vs pressure for (△) 0.76 and (○) 0.076 μM dansyl-*lac* repressor tetramer at 4.5 °C.

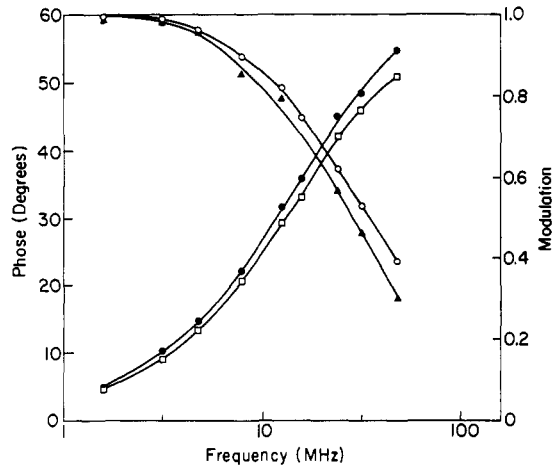

FIGURE 2: Phase and modulation of 0.76 μM dansyl-*lac* repressor at atmospheric pressure (solid symbols) and at 2.2 kbar (open symbols) at 4.5 °C.

was only 15%. The high-pressure lifetimes were measured in this case and are reported under Results. Average lifetime values were consistently measured at atmospheric pressure to be approximately 13 ns at 4.5 °C and 11 ns at 21 °C.

Results

Dissociation of the Dansyl-lac Repressor Tetramer. The pressure dependence of the polarization of the dansyl-*lac* repressor tetramer for two protein concentrations (0.76 and 0.076 μM dansyl-*lac* tetramer) at 4.5 °C is shown in Figure 1. The polarization at atmospheric pressure is equivalent for both solutions. Each solution shows a decrease in polarization as a function of pressure, with the lower concentration responding at lower pressures, as would be expected from mass action. A plateau is reached for this lower concentration, indicating that the observed dissociation transition is complete. The pressure dependence of the frequency response of the dansyl polarization for the 0.76 μM dansyl-*lac* tetramer solution is shown in Figure 2. A slight shift to higher frequencies of the phase shift and demodulation curves is observed at 2.2 kbar. These data were analyzed globally and best fit to a double-exponential decay model in which preexponential values were unlinked between the pressure data sets and lifetime values were linked. This analysis yielded 15.7- and 3.0-ns components. The preexponential factor for the long component decreased by 5% over this pressure range. The average lifetimes calculated from the values of the fractional intensities were 13.5 ns at atmospheric pressure and 12.7 ns at 2 kbar. From the polarization values and the lifetimes at atmospheric pressure and 2.2 kbar, the rotational relaxation time, ρ, was calculated as described under Materials and Methods. At atmospheric pressure, the value of ρ is 126 ns, whereas that calculated at the end of the dissociation transition is 67 ns (Table I). There is little change in viscosity over this pressure range (Bridgeman, 1970), and rotational relaxation times of approximately 30-fold the lifetime value can be calculated with reasonable certainty (Neyroz et al., 1987). More important, however, than the absolute values of the relaxation times are their relative values at low and high pressure. In Table I is also shown the rotational relaxation time calculated from the pressure/polarization data of dansyl-*lac* pressor at a slightly different concentration (0.5 μM) also at 4.5 °C. Again an approximate 2-fold decrease in the relaxation time is observed (from 127 to 58 ns). The tetramer would be expected to have a rotational correlation time approximately twice as large as that observed at atmospheric pressure; however, the local mobility of the dansyl residues likely contributes to the total depolarization (Bandyopadyay et al., 1981). The factor of approximately 2 between the low- and high-pressure values is consistent with a 2-fold decrease in the size of the complex. Since dilution studies reported under Materials and Methods as well as ultracentrifugation studies (Matthews, unpublished results) show no dissociation of the tetramer above 0.5 μM, we conclude that the species present at atmospheric pressure is tetrameric and that the high-pressure species corresponds to the dansyl-*lac* repressor dimer. The equation for the dissociation of a tetramer to a dimer is equivalent to that of a dimer to a monomer. Therefore, the dissociation constant at each pressure was extracted from the relation used by Paladini and Weber (1981a) for the dissociation of the enolase dimer:

$$K_{dp} = \frac{4C_0\alpha_p^2}{1 - \alpha_p} \quad (4)$$

where C_0 is the total protein concentration expressed as tetramer and α_p is the degree of tetramer dissociation at pressure p. Plotting ln $[\alpha^2/(1 - \alpha)]$ vs pressure yields a slope of $\Delta V_a/RT$ and an intercept of ln $K_d/4C_0$ at atmospheric pressure. The recovered values for K_d, ΔG_a, and ΔV_a from the linear regression of the data at 0.76 μM tetramer and also for the 0.5 μM solution at 4.5 °C are given in Table II. The average K_d for the tetramer to dimer transition at 4.5 °C calculated from 0.76 and the 0.5 μM solutions is 4.3 nM. The

Table I: Rotational Relaxation Times

$[lac]_T$ (μM)	T (°C)	[IPTG] (mM)	[operator] (μM)	Q^a	$\rho_{atm}{}^b$ (ns)	$\rho_{2.2kbar}{}^b$ (ns)
0.76	4.5	0.0	0.0	0.87	126	67
0.76	4.5	1.0	0.0	0.85	124	61
0.5	4.5	0.0	0.0	0.86	127	58
0.5	4.5	0.0	0.5	0.90	156	58
0.5	21.0	0.0	0.0	0.91	86	39
0.5	21.0	1.0	0.0	0.94	63	30
0.5	21.0	0.0	0.0	0.92	52	28
0.5	21.0	1.0	0.5	0.94	58	28
0.5	21.0	0.0	1.0	0.94	103	31
0.5	21.0	1.0	1.0	1.01	56	27
2.5 (pH 9.2)	21.0	0.0	0.0	0.92	61	20
0.5 (pH 9.2)	21.0	1.0	0.0	0.87	59	23

^{a} Q is the ratio of the total intensity at atmospheric pressure to that at 2.2 kbar. ^{b} Errors associated with the values of ρ are approximately ±5 ns calculated from the standard deviations in the polarization measurements.

Table II: Thermodynamic Parameters

$[lac]_T$ (μM)	T (°C)	[IPTG] (mM)	K_d (nM)	ΔG_a (kcal/mol)	ΔV_a (mL/mol)
0.76	4.5	0.0	5.6 ± 2.1	−10.5 ± 0.2	80 ± 5
0.76	4.5	1.0	7.1 ± 2.9	−10.3 ± 0.4	77 ± 6
0.50	4.5	0.0	3.0 ± 1.2	−10.7 ± 0.2	86 ± 2
0.50	21.0	0.0	14.0 ± 1.2	−10.6 ± 0.1	88 ± 5
0.50	21.0	1.0	3.4 ± 1.3	−11.3 ± 0.2	88 ± 4
0.50	21.0	0.0	11.5 ± 2.9	−10.7 ± 0.2	93 ± 5

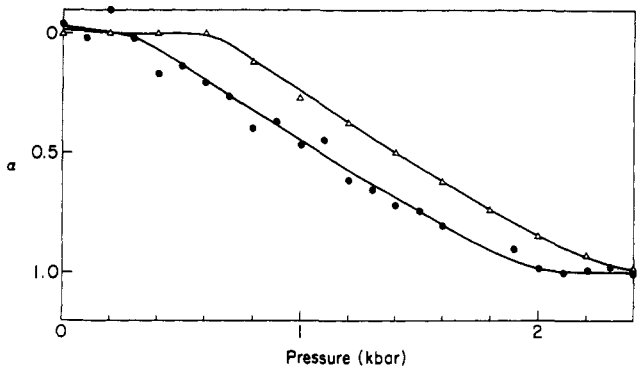

FIGURE 3: Degree of complex dissociation vs pressure for (△) 0.5 μM dansyl-*lac* repressor at 4.5 °C and (●) 0.5 μM dansyl-*lac* repressor at 21 °C.

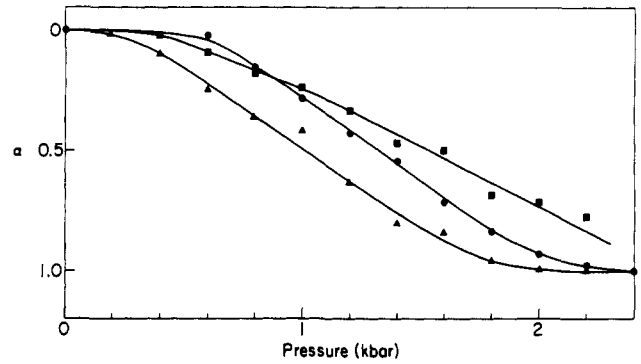

FIGURE 4: Degree of complex dissociation (α) vs pressure for 0.5 μM dansyl-*lac* repressor at 21 °C (▲) alone, (■) in the presence of 1 mM IPTG, and (●) in the presence of 1 mM IPTG and 0.5 μM 40 base pair operator.

operator binding activity of the dansyl-*lac* repressor was measured before and after pressurization. The operator binding equilibrium K_d values calculated for these two solutions were respectively 0.02 and 0.04 nM. Operator affinity for unmodified and unpressured repressor under the same assay conditions was between 0.01 and 0.03 nM. Thus, neither modification with the fluorescent probe nor pressurization had a significant effect upon operator binding by *lac* repressor.

The presence of IPTG had no significant effect on the pressure-induced tetramer dissociation. A small increase in the dissociation constant was observed, from 4.5 to 7.1 nM. However, these values are within the reported error. A comparison between the recovered K_d and ΔG values in absence and in presence of inducer at 4.5 °C (Table II) shows no difference within the reported experimental error. We have shown previously (Royer et al., 1986) that the application of high pressure leads to 90% dissociation of millimolar IPTG by 2.4 kbar. Despite this dissociation, if IPTG binding had either a stabilizing or a destabilizing effect upon the *lac* tetramer, a shift as well as a slope change in the pressure-induced dissociation curve would be anticipated. These anticipated changes are derived from the pressure dependence of the fractional saturation with inducer. If inducer-bound repressor had different subunit affinities than free repressor, the pressure dependence of the protein dissociation profile would be altered in the presence of inducer. This expectation is clearly not borne out.

Effect of Temperature on Repressor Subunit Affinity. The pressure dependence of the degree of tetramer dissociation calculated by using eq 2 from the pressure/polarization data for 0.5 μM dansyl-*lac* repressor at 21 °C is plotted in Figure 3 along with the dissociation profile obtained for this same concentration at 4.5 °C. There is a very large shift to lower pressures of the high-temperature curve. The polarization values for the two solutions at atmospheric pressure were not equivalent due to increased local as well as global rotational mobility at the higher temperature. As in the case of the low-temperature data, there is an approximate 2-fold decrease in the rotational relaxation time from 86 to 39 ns or from 62 to 28 ns depending upon the labeling preparation (Table I), again consistent with a tetramer–dimer transition. The dissociation constants, volume changes, and free energies recovered from the high-temperature data are provided in Table II, along with those already presented for the low-temperature data. There is a 3-fold increase in the dissociation constant from 4.3 to 12.7 nM upon raising the temperature from 4.5 to 21 °C. There is no significant change in the value of the free energy due to the RT term. As a measure of reproducibility, the above experiment was repeated with three separate preparations of dansyl-*lac* repressor. The absolute value of the polarization at atmospheric pressure (0.255, 0.263, 0.274) differs slightly for these three preparations due to small differences in labeling ratios, but the dissociation profiles are identical within error, exhibiting the same total change in polarization and the same $p_{1/2}$ (pressure for 50% dissociation).

The effect of IPTG binding on the pressure/polarization profile was investigated at 21 °C. IPTG binding resulted in a decrease in the polarization at atmospheric pressure of 0.016 polarization unit. Since IPTG binding had no effect upon the fluorescence lifetime and since no evidence for tetramer dissociation by dilution at atmospheric pressure was found either in the presence or in the absence of IPTG, we interpret this decrease to be due to a conformational change which occurs upon IPTG binding and which results in greater local mobility of the dansyl label. Wu and co-workers (Wu et al., 1976; Bandyopadyay et al., 1981) found that the binding of IPTG by the *lac* repressor resulted in a large increase of the local mobility both of the intrinsic trypophan residues and also of a covalently attached fluorescent probe, *N*-[[(iodoacetyl)-amino]ethyl]-5-naphthylamine-1-sulfonate (IAEDANS).

The high-temperature pressure/polarization curves for this 0.5 μM solution in the absence and in presence of IPTG were used to calculate the degree of complex dissociation using eq 2. The plots of α vs pressure for these solutions are shown in Figure 4. A shift of approximately 400 bar to higher pressure is observed for dissociation of the solution in the presence of IPTG. There is thus a stabilizing effect of IPTG on the subunit interactions at 21 °C which is not observed at 4.5 °C. Due to the slight effect of IPTG binding on the polarization, the dissociation profile may reflect IPTG dissociation to some extent. However, the total effect is small (0.016 polarization unit), and there is no evidence for a plateau at high pressures, even though the IPTG should be almost completely dissociated by this pressure (Royer et al., 1986). Thus, the observed shift to higher pressures is interpreted as resulting primarily from tetramer stabilization. The dissociation constant for induced repressor at 21 °C is found to be 3.5 nM, 4-fold lower than in the absence of inducer, corresponding to an increase in the absolute value of the Gibbs free energy from 10.5 to 11.3 kcal/mol (Table II).

Effect of pH on the Repressor Subunit Affinity. In Figure 5 are plotted the pressure polarization profiles of 0.5 μM dansyl-*lac* repressor in the absence and in the presence of 1 mM IPTG at pH 9.2 at 21 °C. When these results are compared to those obtained at neutral pH (replotted in Figure 5 for comparison), it can be seen that the total change in polarization over this pressure range for the high-pH solutions is larger than that found for the dissociation to dimer. In

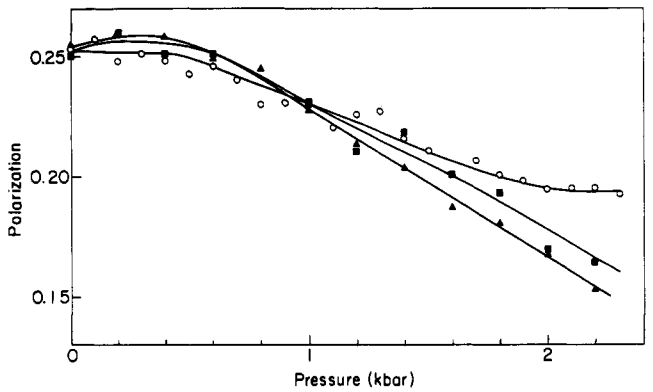

FIGURE 5: Polarization vs pressure for 0.5 μM dansyl-*lac* repressor at 21 °C (○) at pH 7.1, (▲) pH 9.2, and (■) pH 9.2 in the presence of 1 mM IPTG.

addition, no high-pressure plateau is reached. The 3-fold decrease in the rotational relaxation times calculated from the polarization data at high and low pressures (Table I) for the high-pH solutions is indicative of dissociation to monomer, although the transition is clearly not complete by 2.2 kbar. Since no plateau was reached, a dissociation constant could not be calculated for the apparent direct transition to monomer. Inducer binding causes a small stabilization of the subunit interactions as evidenced by the 200-bar shift of the polarization curve to higher pressures. This inducer effect is smaller in amplitude than that observed for the solution at 21 °C and at neutral pH.

Dissociation of the Repressor–Operator Complex. The pressure dependence of the dansyl-*lac* polarization in the complex with a 40 base pair oligonucleotide containing the operator sequence was examined at 4.5 °C. The concentrations used in this experiment were 0.5 μM dansyl-*lac* tetramer and 0.5 μM 40-mer. At these concentrations, all of the operator is bound by repressor, and the repressor should be 50% saturated with the operator, one operator bound per tetramer. Plots of the pressure dependence of the polarization of the tetramer alone and the repressor–operator complex are shown in Figure 6a. A slight increase in the value of the polarization is evident at atmospheric pressure when operator is present. This observation is consistent with complex formation. The rotational relaxation time calculated from the atmospheric polarization values increases from 127 to 156 ns upon addition of operator (Table I). The polarization of the dansyl-*lac* repressor both in the absence and in the presence of the operator DNA decreases as a function of pressure and reaches approximately the same value at high pressure as the 0.5 μM dansyl-*lac* repressor alone, indicating that the average size of the particle is approximately equivalent at 2.2 kbar. Since a higher polarization value would be expected if any tetramer–operator or dimer–operator complex were present, we can conclude that, in both cases, the high-pressure species corresponds predominantly to the dansyl-*lac* dimer and thus that high hydrostatic pressure results in the dissociation of the operator sequence from the repressor protein. Upon examination of the pressure/polarization profile for the dansyl repressor in the presence of operator (Figure 6a), it can be seen that above 1 kbar the values of the polarization in the presence of operator are lower than those obtained in its absence, indicating that the average size of the complex at these pressures is smaller when operator is present than in its absence. The polarization data were transformed into plots of the degree of complex dissociation vs pressure using eq 2 and are shown in Figure 6b. The repressor–operator complex is more easily dissociated to yield free dimer than the tetramer alone. In

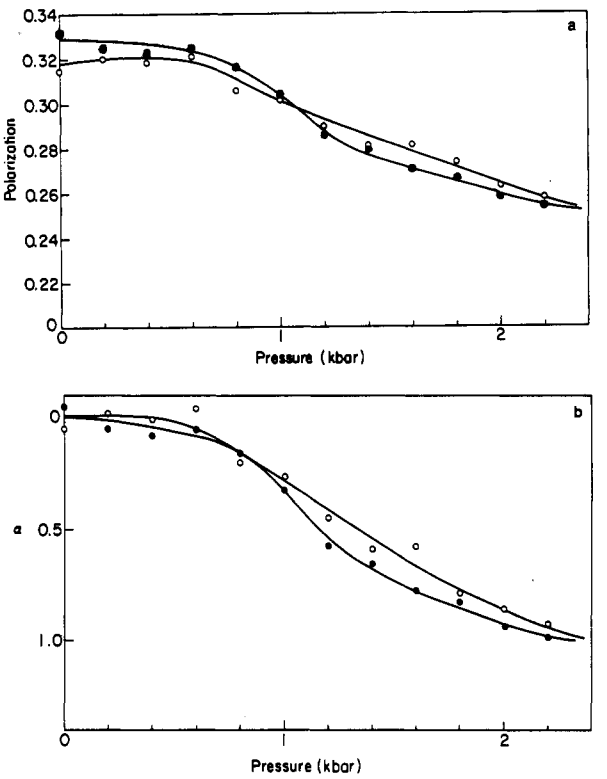

FIGURE 6: (a) Polarization vs pressure for 0.5 μM dansyl-*lac* repressor at 4.5 °C (○) in the absence and (●) in the presence of 0.5 μM 40 base pair operator. (b) Degree of complex dissociation, α, vs pressure calculated from the data in (a) and eq 2 (○) in the absence and (●) in the presence of 0.5 μM 40 base pair operator.

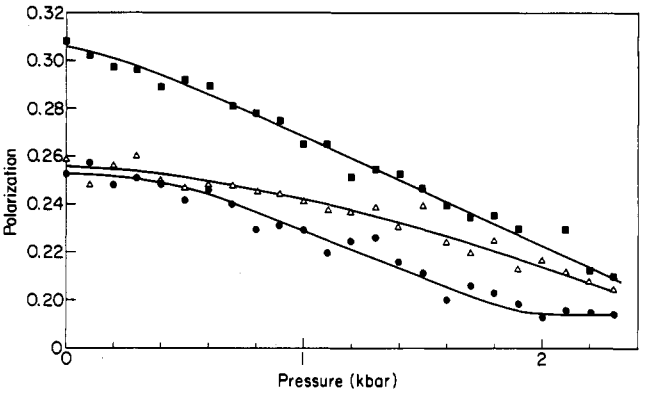

FIGURE 7: Polarization vs pressure for 0.5 μM dansyl-*lac* repressor at 21 °C (●) Alone; (■) in the presence of 1 μM 40 base pair operator; (△) in the presence of 1 μM 40 base pair operator and 1 mM IPTG.

addition, two slopes are evident in the dissociation profile of the repressor–operator complex, which we interpret as corresponding to subunit dissociation and operator dissociation. Comparing the shapes of the polarization curves in Figure 6a, it appears that the second slope may be more highly weighted for tetramer dissociation whereas the first may be more indicative of operator dissociation. Because the measured polarization is an average of that of all of the possible species in solution (operator-bound tetramer, operator-bound dimer, free dimer, free tetramer), it is not possible to give a quantitative interpretation to this complex dissociation profile at this time. However, it is possible that the lower polarization values observed above 1 kbar in the presence of operator stem from a smaller molecular volume for operator-bound dimer.

The effect of pressure on the dansyl-*lac* repressor–operator complex was examined at 21 °C under conditions of saturating operator, 0.5 μM tetramer, and 1 μM operator, two operator

molecules per tetramer. The results are shown in Figure 7. It can be seen that there is a large increase in the polarization of the dansyl-*lac* repressor at atmospheric pressure when saturating operator is present compared to repressor alone, consistent with complex formation under the conditions of this experiment. The polarization of the complex decreases immediately upon pressurization, indicating great ease in complex and/or subunit dissociation. As in the previous experiment, the value of the polarization of the repressor in the presence of operator at 2.2 kbar is almost the same as that measured for the solution of repressor alone, indicating that dansyl-*lac* repressor dimer is the predominant high-pressure species.

Effect of Pressure on the Induced Repressor–Operator Complex. When IPTG at a concentration of 1 mM, as well as half-saturating operator (Figure 4), is present at 21 °C, the polarization at atmospheric pressure is lower than that of the repressor alone, 0.27 compared to 0.29. We again interpret this as due to an IPTG-induced conformational change which increases the local mobility of the dansyl residues (Wu et al., 1976; Bandyopadyay et al., 1981). The pressure/polarization profile for the ternary complex at 50% operator saturation and 1 mM IPTG is found to be intermediate between that measured for the repressor in the presence of inducer alone and that observed for the repressor in the absence of any ligand. Upon addition of 1 mM inducer to a 0.5 μM dansyl-*lac* solution containing 100% saturating 40 base pair operator (1 μM) at 21 °C (Figure 7), the value of the polarization at atmospheric pressure is found to be nearly equal to that found for the repressor alone, again interpreted as resulting from an IPTG-induced conformational change affecting the local mobility of the dansyl residue. The complete dissociation at atmospheric pressure of the repressor–operator complex upon addition of IPTG would not be expected at such a high operator concentration which permits even nonspecific binding. At all pressures, the observed polarization values for the solution containing both inducer and operator are intermediate between those obtained for the repressor alone and those observed in the presence of saturating operator. The results of these two experiments on the ternary complex can be understood in light of the concomitant interplay between the destabilizing effect of pressure on all of the binding equilibria and the antagonism between operator and IPTG binding. Since pressure results in the dissociation of the inducer sugar as well as the operator, their relative affinities are a complex result of their differential volume effects. IPTG binding at 21 °C has the effect of decreasing the absolute value of the polarization while stabilizing the repressor tetramer. Operator binding has the opposite effect. Due to the size of the 40 base pair fragment, the polarization value increases upon binding, whereas the complex is more easily dissociated by pressure. Thus, the pressure effects on the ternary complex are intermediate between those obtained for the free repressor and those for the repressor in the presence of operator or inducer alone.

Discussion

The combination of two techniques, exposure to high hydrostatic pressure and fluorescence spectroscopy, allows measurement of *lac* repressor tetramer dissociation to dimer using a concentration which is 2 orders of magnitude larger than the dissociation constant. The value of the free energy of the dissociation reaction at 4.5 °C was found to be 10.5 ± 0.2 kcal/mol, corresponding to a dissociation constant of 4.3 ± 1.3 nM. We have observed a large effect of temperature on the dissociation profile of the repressor, with the dissociation constant increasing by a factor of 3 between 4.5 and 21 °C. The presence of IPTG stabilizes the tetramer at high temperature (4.5-fold decrease in the dissociation constant corresponding to 0.8 kcal/mol), whereas at low temperature, the subunit affinity in the induced repressor is not altered compared to that found for repressor alone. Chakerian and coworkers (Chakerian et al., 1987) observed a transition in the Arrhenius plots of IPTG association rates at 12 °C. Thus, it appears that the conformational transition responsible for the altered activation energy and association rate for IPTG binding also modulates the interactions at the subunit interface as well as the coupling between inducer binding and subunit interactions. Whitson and co-workers (Whitson et al., 1986) have shown that operator binding is temperature dependent as well, with maximal affinity found at approximately 12 °C and a decrease in affinity as the temperature is either raised or lowered. These results were analyzed by Ha et al. (1990) in their treatment of a number of protein–DNA interactions which exhibit a characteristic maximum for the K_d in this temperature range. They concluded that a large hydrophobic surface area must be buried upon association and that this removal of hydrophobic protein residues from the solvent is a major driving force for the binding process. The observed binding affinity in these systems results from the temperature profiles of more than one equilibrium process. Our results suggest that the subunit interface may be partially responsible for the observed temperature effects on binding parameters for the dansyl-*lac* repressor.

High pH was also found to alter the dissociation profile. Although a shift of the profile to lower pressure was not observed, the transition showed a steeper slope, a larger total change over the standard pressure range, and no evidence for a high-pressure plateau. From this, we conclude that at high pH, the transition is a concerted transition to monomer which is only partially ($\simeq$75%) complete by 2.2 kbar. The destabilization of the tetrameric structure at high pH is consistent with the apparent destabilization in the presence of operator DNA. Previous data (Daly & Matthews, 1986a) have indicated that the high-pH form of the repressor has lower affinity for inducer relative to the neutral pH form. In addition, inducer binding is cooperative at pH 9.2. The vicinity of residue 281 is thought to be responsible for the pH dependence of inducer binding and has been implicated in subunit interactions as well (Daly & Matthews, 1986b; Daly et al., 1986; Royer et al., 1986).

The application of high hydrostatic pressure results in the destabilization of the dansyl-*lac* repressor–operator interactions. The concentration of operator used in these experiments was approximately 10^5-fold over the value of the K_d for operator binding (10^{-11} M). In order to obtain an estimate for the value of the volume change upon association, ΔV_a, necessary for the observation of pressure-induced operator dissociation, one can assume a simple protein–ligand equilibrium. The volume change can then be calculated as follows (Royer et al., 1986):

$$\ln (L_0/K_d) = (p_{1/2}/RT)\Delta V_a \qquad (5)$$

where L_0 is the total ligand concentration and $p_{1/2}$ is the pressure for 50% dissociation. Assuming that 1.5 kbar is the 50% dissociation point, one can calculate that the volume change upon association needed for such a reaction is approximately 200 mL/mol. Although this value is not excessively large (typical volume changes for tetramer to monomer transitions are similar) (King & Weber, 1986), it is indicative of a relatively large amount of buried surface area upon operator binding. A large volume change upon operator binding is also consistent with the recent interpretation of the temperature dependence studies of DNA binding proteins by Ha

and co-workers (Ha et al., 1990). Electrostriction could play a role in the negative volume change for repressor–operator interactions. However, one would expect that at 100 mM in KCl, charge–charge interactions between the nucleic acid and the protein would simply be replaced by the counterions of the salt. We suggest, therefore, that the protein–nucleic acid interface is a "hard-site", following the definition of Torgerson and co-workers (Torgerson et al., 1979), with a relatively large excluded volume which can be filled with solvent molecules in the dissociated form. It is well-known (Manning, 1978) that the DNA helix presents a high degree of hydration in solution. In addition, burial of a large surface area of the protein upon binding will not result in perfect packing, hence, the exclusion of even more solvent. The combination of the dehydration of the protein and the DNA surfaces results in the observed increase in the volume of the system upon association because water molecules occupy more volume in solution than when tightly bound to the macromolecular surfaces.

One implication derived from the equilibrium constants for tetramer dissociation calculated from our pressure data is that at the low protein concentrations required for binding assays of *lac* repressor affinity for operator DNA, the protein is not tetrameric. The presence of DNA may influence this equilibrium, particularly DNA with multiple operator sites or extensive nonspecific sequences, as the effective concentration of repressor bound to such sequences is larger than that for bulk solution. In this regard, it is noteworthy that a mutant dimeric *lac* repressor has been described by Lehming and co-workers (Lehming et al., 1988) with somewhat lower affinity compared to the tetrameric repressor for *lac* operator in linear DNA. Thus, the tetramer/dimer equilibrium may have a minimal effect on the observed affinity of the repressor for DNA sequences containing a single operator. However, the requirement for tetrameric protein in DNA loops involving multiple operator sites (Mossing & Record, 1986; Whitson, et al., 1987a,b; Kramer et al., 1987, 1988) suggests that binding in this mode, which is demonstrable in vivo (Borowiec et al., 1987; Sasse-Dwight & Gralla, 1988; Flashner & Gralla, 1988; Bellomy et al., 1988), may be considerably influenced by the relative population of tetrameric and dimeric states of the repressor. The superposition of the protein subunit equilibria and protein–operator binding would thus determine the occupation of a specific operator site and thereby influence the extent of transcription.

Finally, we have observed that the dansyl-*lac* repressor–operator complex is apparently more easily destabilized by pressure than the dansyl-*lac* tetramer alone. Due to the superposition of multiple equilibria (dimer–dimer, tetramer–operator, and dimer–operator) in these experiments, we cannot conclude which species are present at intermediate pressures from the polarization data alone. Our results, however, are highly suggestive of a negative free energy coupling between operator binding and subunit interactions. If operator DNA binding had a stabilizing effect upon the tetramer, then one would expect to see a long plateau before pressure-induced dissociation would occur. Instead, for the 100% saturated complex, we observe an immediate decrease in the polarization upon the application of pressure. At lower temperature and 50% saturation, a plateau is evident, but it is no longer than that observed in the absence of operator. In addition, the complex is more easily dissociated in the presence of operator than in its absence, despite the fact that low temperature stabilizes tetramer interactions. The apparent formation of monomer at high pH supports the hypothesis that operator destabilizes the tetramer interface, as high pH has been shown to mimic the high operator affinity state of the protein (Daly & Matthews, 1986).

In summary, measurement of the pressure dependence of the polarization of dansyl-*lac* repressor under a variety of solution conditions indicates that the protein dissociates to a dimeric form at neutral pH and proceeds partially to monomer at elevated pH values. The operator-bound complex is more easily destabilized than the tetramer alone, consistent with previous observations that suggest a similarity between the high-pH conformation and the operator-bound form of this protein (Daly & Matthews, 1986). IPTG had no appreciable effect upon the subunit interactions at low temperature, but near room temperature, the subunit interactions are stabilized by the presence of the sugar ligand. The superposition of the multiple equilibria in this system at concentrations found in the cell indicates significant coupling between subunit interactions, operator binding, and inducer affinity to determine the consequent expression of the genes in the *lac* operon. This strategy of coupling subunit affinity to ligand affinity to determine binding density may be a common phenomenon in both prokaryotic and eukaryotic systems.

Registry No. IPTG, 367-93-1.

References

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Bandyopadyay, P. K., Wu, F. Y.-H., & Wu, C.-W. (1981) *J. Mol. Biol. 145*, 363–373.

Barkley, M. D., Riggs, A. D., Jobe, A., & Bourgeois, S. (1975) *Biochemistry 14*, 1700–1712.

Bellomy, G. R., Mossing, M. C., & Record, M. T., Jr. (1988) *Biochemistry 27*, 3900–3906.

Borowiec, J., Zhang, L., Sasse-Dwight, S., & Gralla, J. (1987) *J. Mol. Biol. 196*, 101–110.

Chakerian, A. E., Olson, J. S., & Matthews, K. S. (1987) *Biochemistry 26*, 7250–7255.

Collen, D., Vandereycken, G., & DeMaeyer, L. (1970) *Nature 228*, 669–671.

Daly, T. J., & Matthews, K. S. (1986a) *Biochemistry 25*, 5479–5484.

Daly, T. J., & Matthews, K. S. (1986b) *Biochemistry 25*, 5474–5478.

Daly, T. J., Olson, J. S., & Matthews, K. S. (1986) *Biochemistry 25*, 5468–5474.

Flashner, Y., & Gralla, J. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8968–8972.

Gilbert, W., & Müller-Hill, B. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 1891–1898.

Gratton, E., & Limkeman, M. (1983) *Biophys. J. 44*, 315–323.

Ha, J.-H., Spolar, R. S., & Record, M. T., Jr. (1990) *Biochemistry* (in press).

Heremans, K. A. H. (1974) in *Proceedings of the 4th International Conference on High Pressure*, Kyoto, Japan, pp 627–630.

Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.

Hsieh, W. T., & Matthews, K. S. (1985) *Biochemistry 24*, 3043–3049.

Jaenicke, R., Ludemann, H.-D., & Schade, B. C. (1981) *Biophys. Struct. Mech. 7*, 195–203.

King, L., & Weber, G. (1986) *Biochemistry 25*, 3632–3637.

Kramer, H., Niemoller, M., Amouyal, M., Revet, B., von-Wilcken-Bergmann, B., & Müller-Hill, B. (1987) *EMBO J. 6*, 1481–1491.

Kramer, H., Amonyal, M., Nordheim, A., & Müller-Hill, B. (1988) *EMBO J. 7*, 547–556.

Lehming, N., Sartorius, J., Oehler, S., vonWilken-Bergmann, B., & Müller-Hill, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7947-7951.
Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179-246.
Mossing, M. C., & Record, M. T., Jr. (1986) *Science 233*, 889-892.
Miller, J. H., & Reznikoff, W. S. (1980) in *The Operon* (Miller, J. H., & Reznikoff, W. S., Eds.) 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Müller, K., Ludeman, H.-D., & Jaenicke, R. (1981) *Biophys. Chem. 14*, 101-110.
Neyros, P., Brand, L., & Roseman, S. (1987) *J. Biol. Chem. 262*, 15900-15907.
O'Gorman, R. B., Dunaway, M., & Matthews, K. S. (1980a) *J. Biol. Chem. 255*, 10100-10106.
O'Gorman, R. B., Rosenberg, J. M., Kallai, O. B., Dickerson, R. E., Itakura, K., Riggs, A. D., & Matthews, K. S. (1980b) *J. Biol. Chem. 255*, 10107-10114.
Paladini, A. A., & Weber, G. (1981a) *Biochemistry 20*, 2587-2593.
Paladini, A. A., & Weber, G. (1981b) *Rev. Sci. Instrum. 52*, 419-427.
Payens, T. A. J., & Heremans, K. (1969) *Biopolymers 8*, 335-345.
Perrin, F. (1926) *J. Phys. Radium 1*, 390-401.
Perutz, M. (1972) *Nature 237*, 495-499.
Riggs, A. D., Bourgeois, S., Newby, R. F., & Cohn, M. (1968) *J. Mol. Biol. 34*, 365-368.
Riggs, A. D,. Suzuki, H., & Bourgeois, S. (1970a) *J. Mol. Biol. 48*, 67-83.
Riggs, A. D., Bourgeois, S., & Cohn, M. (1970b) *J. Mol. Biol. 53*, 401-417.
Rosenberg, J. M., Kallai, O. B., Kopka, M. L., Dickerson, R., & Riggs, A. D. (1977) *Nucleic Acids Res. 4*, 567-572.
Royer, C. A., Weber, G., Daly, T. J., & Matthews, K. S. (1986) *Biochemistry 25*, 8308-8315.
Ruan, K., & Weber, G. (1988) *Biochemistry 27*, 3295-3301.
Sasse-Dwight, S., & Gralla, J. D. (1988) *J. Mol. Biol. 202*, 107-119.
Silva, J. L., Miles, E. W., & Weber, G. (1986) *Biochemistry 25*, 5781-5786.
Suzuki, K., & Tanigachi, T. (1972) in *The Effects of Pressure on Organisms* (Sleigh, M. A., & MacDonald, A. G., Eds.) pp 103-124, Cambridge University Press, Cambridge.
Torgerson, P. M., Drickamer, H. G., & Weber, G. (1979) *Biochemistry 18*, 3079-3083.
von Hippel, P. H., & McGhee, J. D. (1972) *Annu. Rev. Biochem. 41*, 231-300.
Weber, G., & Drickamer, H. (1983) *Q. Rev. Biophys. 116*, 89-112.
Whitson, P. A., & Matthews, K. S. (1986) *Biochemistry 25*, 3845-3852.
Whitson, P. A., Olson, J. S., & Matthews, K. S. (1986) *Biochemistry 25*, 3852-3858.
Whitson, P. A., Hsieh, W. T., Wells, R. D., & Matthews, K. S. (1987a) *J. Biol. Chem. 262*, 4943-4946.
Whitson, P. A., Hsieh, W. T., Wells, R. D., & Matthews, K. S. (1987b) *J. Biol. Chem. 262*, 14592-14599.
Wu, F. Y.-H., Banyopadyay, P., & Wu, C.-W. (1976) *J. Mol. Biol. 100*, 459-472.

Regulation of Plasminogen Activation by Components of the Extracellular Matrix†

Sharon Stack, Mario Gonzalez-Gronow, and Salvatore V. Pizzo*
Departments of Pathology and Biochemistry, Box 3712, Duke University Medical Center, Durham, North Carolina 27710
Received December 27, 1989; Revised Manuscript Received February 2, 1990

ABSTRACT: The kinetics of activation of Glu-plasminogen (Glu-Pg) and Lys_{77}-Pg by two-chain recombinant tissue plasminogen activator (t-PA) were determined in the presence of isolated protein components of the extracellular matrix (ECM) and compared to activation in the presence of fibrinogen and fibrinogen fragments and in the absence of added protein. Several ECM protein components were as effective as fibrinogen fragments at stimulating Pg activation. Stimulation of Glu-Pg activation resulted from both a decrease in K_m and an increase in V_{max}, whereas stimulation of Lys_{77}-Pg was due primarily to increases in V_{max}. The most effective stimulators of activation were basement membrane type IV collagen and gelatin which resulted in a 21- and 55-fold increase, respectively, in the k_{cat}/K_m of Glu-Pg (relative to a 10-fold increase observed with fibrinogen fragments). Amidolytic activity of t-PA was also enhanced up to 12-fold by ECM proteins. However, plasmin amidolytic activity was unaffected by the presence of added proteins. These data suggest that several ECM-associated proteins can enhanced the activation of Pg in the absence of fibrin.

Plasminogen (Pg)[1] is the circulating zymogen form of the serine proteinase plasmin (Pm). Conversion of Pg to Pm is catalyzed by a number of plasminogen activators [for a review, see Castellino (1981)]. Activation of Pg by tissue plasminogen activator (t-PA) occurs very slowly in systems containing only Pg and t-PA (Hoyalerts et al., 1982). However, several studies have demonstrated that Pg activation is stimulated when the zymogen, activator, or both are immobilized by binding to a macromolecular protein surface such as fibrin (Ranby, 1982) or CNBr fragments of fibrinogen (Nieuwenheuizen, 1983).

† This work was supported by Research Grants HL-31939 and HL-43339 from the National Heart, Lung and Blood Institute.
* To whom correspondence should be addressed at the Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710.

[1] Abbreviations: Pg, plasminogen; t-PA, tissue plasminogen activator; ECM, extracellular matrix; TSP, thrombospondin; HRGP, histidine-rich glycoprotein; VLK-pNA, D-Val-Leu-Lys-*p*-nitroanilide; IPR-pNA, D-Ile-Pro-Arg-*p*-nitroanilide; BSA, bovine serum albumin; FN, fibronectin; LN, laminin-nidogen; Pm, plasmin.

0006-2960/90/0429-4966$02.50/0