Effect of Hydrostatic Pressure upon Ethidium Bromide Association with Transfer Ribonucleic Acid†

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ABSTRACT: The binding of ethidium bromide to yeast phenylalanine-specific transfer ribonucleic acid ($tRNA^{Phe}$) has been investigated in the pressure range from 1 atm to 9 kbar in the presence of 100 mM sodium chloride and 10 mM magnesium chloride, pH 7.7. One high-affinity binding site for ethidium is present, with a dissociation constant of 2.4×10^{-6} M at 1 atm and 22 °C. Binding to this site is enhanced with increasing pressure, the dissociation constant reaching 2.9×10^{-7} M at 2 kbar. Pressure also promotes the binding of ethidium to lower affinity sites of $tRNA^{Phe}$. The standard volume change upon complex formation is found to be 25.6 ± 2.7 mL/mol for the first ethidium bound. If sodium is replaced by lithium in the buffer, the standard volume change is 23.3 ± 0.5 mL/mol. We conclude that decrease of the electrostatic repulsion in the negatively charged $tRNA^{Phe}$ by binding of the positively charged ethidium is the main cause of the relatively large volume decrease upon complex formation. The electrostatic repulsion that must be present in this case, as well as in other nucleic acids, implies that intercalating binding sites are of the "soft" type as previously defined [Torgerson, P. M., Drickamer, H. G., & Weber, G. (1979) *Biochemistry 18*, 3079]. Model studies by others of the binding site characteristics are in agreement with this concept.

In recent years a series of observations has been made on the effect of hydrostatic pressures up to 11 kbar upon the binding of small ligands to macromolecules, in particular proteins (Li et al., 1976a,b; Zipp & Kauzmann, 1973; Visser et al., 1977a). Depending upon the system, binding can be either enhanced or destabilized by pressure, but in all the above cases, the observed spectroscopic changes could be interpreted unequivocally as alterations in affinity between the ligand and macromolecule. However, due to the inherent complexity of the systems under consideration, it was difficult to correlate the observed effects with the many processes that can affect the volume of the system, for instance, changes in degree of ionization, exposure to solvent and relative compressibilities. Attempts have been made, therefore, to investigate simpler situations. For instance, Weber et al. (1974) found a ΔV of −4.6 mL for the intramolecular isoalloxazine–adenine complex in flavin adenine dinucleotide, and Visser et al. (1977b) reported a ΔV of −4.8 mL for the association of flavin and indole separated by a five-carbon chain. Interactions between two aromatic ring systems therefore appear to occur with a small decrease in volume.

The interaction of intercalating drugs like ethidium and nucleic acids involves an association between the aromatic rings of the drug and the nucleic acid bases. In the absence of any other effects this might be expected to lead to a decrease in volume on complex formations of a few milliliters per mole. Deviation from this value can give an indication of the participation of the macromolecular structure on the pressure effects.

The details of this interaction have been investigated in general by several workers (Sobell et al., 1976; Nuss et al., 1979), and the proposed models indicate a substantial change in the binding site geometry to accept the ligand. For the present study the specific system chosen was the drug ethidium bromide binding to yeast phenylalanine-specific transfer ribonucleic acid ($tRNA^{Phe}$),[1] since the $tRNA^{Phe}$ offers a well-defined structure to which ethidium binds stoichiometrically with a large fluorescent enhancement. Several investigations of this system have been carried out at atmospheric pressure (Urbanke et al., 1973; Olmsted & Kearns, 1977; Wells & Cantor, 1977; Tao et al., 1970; Bittman, 1969; Jones & Kearns, 1975). The goals of the present study are therefore to determine the type and magnitude of the effect of hydrostatic pressure on the tRNA–ethidium complex and to relate the observed effects to atmospheric data on the complex and to previous pressure-binding data on other systems.

Materials and Methods

Ethidium bromide (Sigma) showed only a single spot on thin-layer chromatography (4:1:1 butanol–acetic acid–water) and was used without further purification. Phenylalanine-specific transfer ribonucleic acid from brewer's yeast was a product of Boehringer Mannheim.

Emission spectra at atmospheric pressure were measured on the spectrofluorometer described by Jameson et al. (1977). For measurements in the range of 1 atm to 2 kbar this instrument was adapted to the pressure cell of Paladini (A. Paladini and G. Weber, unpublished results). Emission data up to 10 kbar were collected as described by Li et al. (1976a,b) and Okamoto (1974), and fluorescence lifetimes in the same pressure range were determined using the single-photon counting apparatus of Klick et al. (1977). Absorption values were obtained on a Beckman Acta MKV1 spectrophotometer using extinction coefficients of 5.4×10^5 at 260 nm for $tRNA^{Phe}$ and 5.3×10^3 at 480 nm for ethidium bromide (Hudson & Jacobs, 1975).

Binding experiments were carried out in buffers containing 10 mM magnesium chloride, 40 mM tris(hydroxymethyl)-aminomethane, pH 7.7, and either 100 mM sodium chloride or 100 mM lithium chloride. Measurements at 1 atm and up to 2 kbar were done at 22 °C, and measurements to 10 kbar were done at room temperature. Emission intensities used to

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[1] Abbreviation used: $tRNA^{Phe}$, phenylalanine-specific transfer ribonucleic acid from brewer's yeast.

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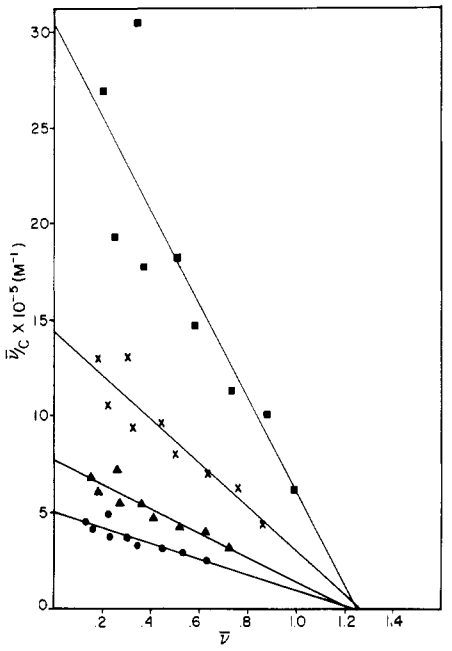

FIGURE 1: Scatchard plots of ethidium bromide binding to 2.6×10^{-6} M $tRNA^{Phe}$ at various pressures: (●) 1 atm; (▲) 0.41 kbar; (×) 1.00 kbar; (■) 1.67 kbar. Buffer contains 100 mM sodium chloride.

produce Scatchard plots as a function of pressure were obtained by measuring the intensity at 1 atm of samples containing a known concentration of $tRNA^{Phe}$ and ethidium bromide, followed by loading individual samples into the pressure apparatus and measuring the change in intensity with increasing pressure. All intensities and concentrations are corrected for compression of the solvent.

Results

For a system such as ethidium–tRNA where association is accompanied by an increase in the emission intensity, the fraction of the ligand bound, β, can be determined by eq 1,

$$\beta = (I_{obsd} - I_{free})/(I_\infty - I_{free}) \quad (1)$$

where I_{free} is the intensity of ethidium bromide emission in the absence of tRNA, I_∞ is the emission intensity upon binding saturation, and I_{obsd} is the intensity in the presence of a known amount of tRNA. Knowledge of β, the ethidium bromide concentration, and the tRNA concentration permits the use of Scatchard relation (eq 2). The results of an atmospheric

$$\bar{\nu}/C = K(n - \bar{\nu}) \quad (2)$$

pressure titration in the sodium-containing buffer are shown in Figure 1, indicating the number of binding sites n is 1.2 ± 0.2 and the dissociation constant K_d is $2.4 \pm 0.5 \times 10^{-6}$ M. Titrations at three representative hydrostatic pressures are also shown in Figure 1. As pressure increases, the intercept on the $\bar{\nu}$ axis remains constant, indicating there is no change in the stoichiometry of the binding. The slope of the line, however, becomes steadily steeper, showing that the affinity increases with pressure. The dissociation constants are 1.6×10^{-6}, 8.8×10^{-7}, and 4.1×10^{-7} M at 0.41, 1.00, and 1.67 kbars, respectively.

The standard volume change upon dissociation of the complex can then be calculated using eq 3, where K_p is the dissociation constant as a function of pressure for the first 2 kbars.

$$\Delta V^\circ = -RT\frac{d \ln K_p}{dp} \quad (3)$$

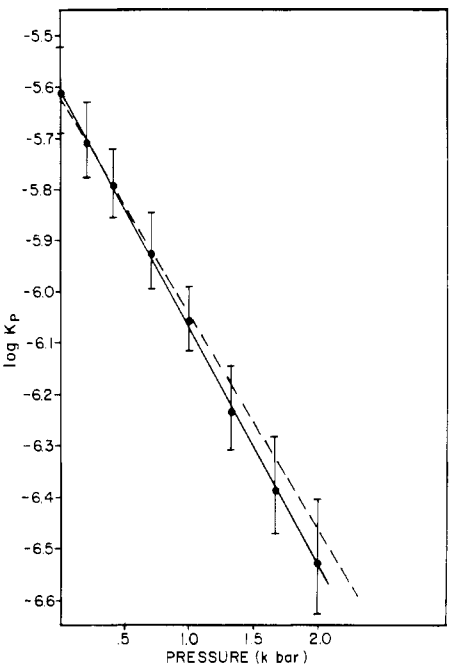

FIGURE 2: Dissociation constant vs. pressure for ethidium bromide binding to $tRNA^{Phe}$: (●) in 100 mM NaCl, 10 mM $MgCl_2$, and 40 mM Tris, pH 7.7; (---) in 100 mM LiCl, 10 mM $MgCl_2$, and 40 mM Tris, pH 7.7.

A plot of log K_p vs. pressure is shown in Figure 2, and yields a standard volume change upon dissociation of +25.6 ± 2.7 mL/mol, equivalent to a free-energy change with pressure of 0.63 kcal/kbar. In other words, increasing the pressure by 2.2 kbar causes a one order of magnitude change in the equilibrium constant. The error bars shown are obtained by calculating the standard deviation of the slopes of the lines in Figure 1. Most of the deviations from the expected straight lines in Figure 1 arise from errors in the preparation of the various solutions and are systematically propagated with equal signs at all pressures. The deviation of the slope in Figure 2, and hence the error in the ΔV°, is therefore calculated using the standard deviations of each point. Figure 2 also shows the results obtained when lithium is substituted for sodium in the buffer. In this case the equilibrium constant at 1 atm is $2.3 \pm 0.1 \times 10^{-6}$ M and the standard volume change upon dissociation is 23.3 ± 0.5 mL/mol.

Results over the pressure range 0 to 9 kbar in the sodium-containing buffer are shown in Figure 3. In the presence of sufficient $tRNA^{Phe}$ to drive binding to over 98% saturation, the intensity is seen to decrease slightly over this range. With lower $tRNA^{Phe}$ concentrations the fluorescence intensity increases until all the ethidium is bound and then behaves identically with the saturated sample. As expected, samples that are further from saturation at 1 atm require higher pressures to drive the binding to completion. Fluorescence lifetimes were also measured on a sample similar to that which showed the largest intensity change in Figure 3. At 1 atm the lifetime of ethidium bromide is 1.8 ns in aqueous solution and 25 ns when bound to $tRNA^{Phe}$. Since the flashlamp in the single-photon counting instrument has a full width at half-height of 3 ns, the data were analyzed only for the longer component. Results are shown in Figure 3. Even though the emission intensity of this sample nearly doubles under pressure, the observed lifetimes correspond exactly to the intensity of the sample with saturating $tRNA^{Phe}$. Therefore, the only effect of pressure on the emission of bound ethidium is that shown by the latter sample and all intensity changes can be inter-

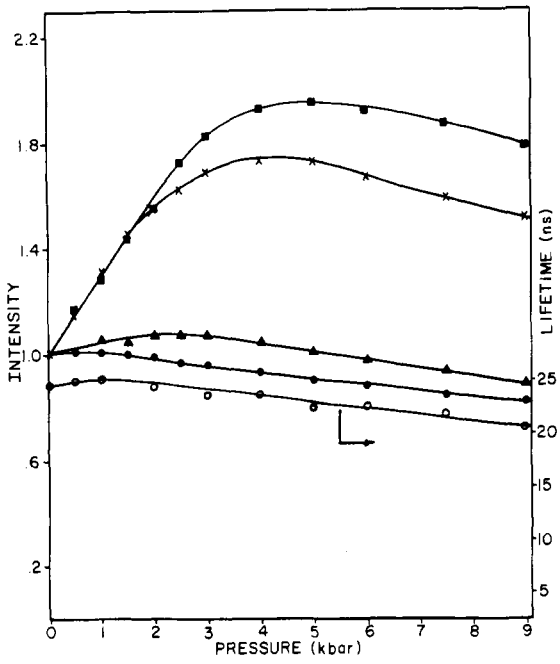

FIGURE 3: Fluorescence intensity and lifetime pressure response for $tRNA^{Phe}$ plus 1.3×10^{-6} M ethidium bromide in sodium-containing buffer. Intensity in the presence of (●) 1.36×10^{-4} M tRNA, (▲) 1.41×10^{-5} M tRNA, (×) 2.6×10^{-6} M tRNA, and (■) 1.64×10^{-6} M tRNA; (○) lifetime in the presence of 1.76×10^{-6} M tRNA.

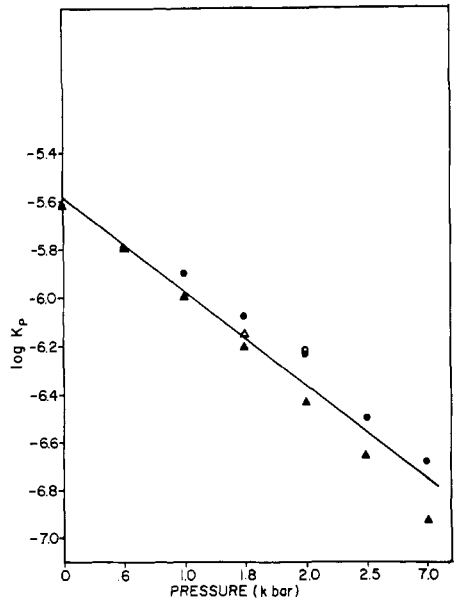

FIGURE 4: Dissociation constant vs. pressure from Figure 3: (●) 1.64×10^{-6} M tRNA; (▲) 2.6×10^{-6} M tRNA; (○,△) data obtained upon releasing and reraising pressure.

preted in terms of shifts in binding equilibria.

Assuming a 1:1 complex, the data of Figure 3 can be analyzed using eq 4, where [X] is ligand concentration, X_t is total

$$K_p = \frac{[tRNA][X]}{[tRNA{\cdot}X]} = \frac{1-\beta}{\beta}[tRNA] = \frac{1-\beta}{\beta}\left(\frac{tRNA_t - \beta X_t}{V_p}\right) \quad (4)$$

ligand, $tRNA_t$ is total tRNA in moles, and V_p is the volume of the system at pressure p relative to the volume at 1 atm. The results are shown in Figure 4 and yield a ΔV of +21.5 ± 1.5 mL. The change in affinity is completely reversible upon releasing pressure.

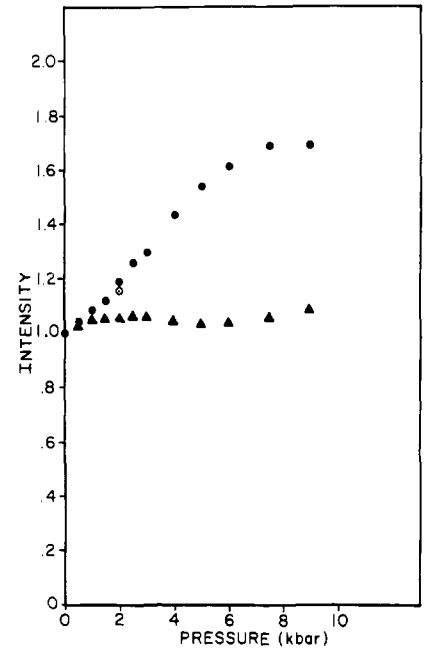

FIGURE 5: Intensity vs. pressure for 2.44×10^{-5} M ethidium plus 2.66×10^{-6} M tRNA: (▲) predicted response for 1:1 binding; (●) observed response; (⊙) observed response upon pressure release.

The preceding experiments were all carried out with at most a small excess of ethidium bromide over $tRNA^{Phe}$. Depending on conditions, $tRNA^{Phe}$ is known to be capable of binding more than one ethidium bromide, although with much lower affinity (Urbanke et al., 1973). Once the emission behavior of free and bound ethidium bromide and the equilibrium constant of the high affinity site are known as a function of pressure, it is possible to calculate the expected emission response knowing only the total amounts of ethidium and $tRNA^{Phe}$ and the volume of the system (eq 4 and 1). The observed and calculated intensities for a ninefold excess of ethidium over tRNA are shown in Figure 5. The excess observed emission is most likely due to secondary ethidium sites whose affinity has been increased by pressure to the point where additional ligand can be bound.

Discussion

The previous results have shown that binding of the first ethidium molecule to $tRNA^{Phe}$ occurs with a decrease in volume of the system of 21 to 26 mL. Previous studies (Weber et al., 1974; Visser et al., 1977a,b; Kasarda, 1970) have shown that association of two aromatic ring systems occurs with a small (2 to 5 mL) decrease in volume. Since in the case of the $tRNA^{Phe}$–ethidium interaction an aromatic ring is removed from the solvent and interacts with two nucleic acid bases, one can reasonably expect a contribution to ΔV of no more than 5 to 10 mL from this effect alone. The remainder of the observed ΔV, then, must come from effects of the binding upon the $tRNA^{Phe}$ molecule itself.

All nucleic acid structures are subject to electrostatic repulsion from the backbone phosphate groups. These effects are partially compensated by counterions in the solvent but will still be operative to some extent through the medium of lower dielectric constant furnished by the bases. It seems reasonable to attribute at least some of the large decrease in volume from ethidium binding to the partial neutralization of the normal electrostatic repulsion by the positive charge introduced in the immediate vicinity of the bases. In addition, studies on the action of intercalating drugs on nucleic acid structure indicate that binding is accompanied by large al-

terations in base-pair structure (Sobell et al., 1976; Nuss et al., 1979). Nuss investigated the effect of ethidium and other dyes on the base-paired dinucleotides GpC and CpG and demonstrated that intercalation is accompanied by a change in the puckering of the ribose rings, a change in the dihedral angles of the sugar–phosphate backbone, and an increase in the distance between the bases of 3.4 Å. Although no work of this type has been done on $tRNA^{Phe}$, it is plausible to assume similar distortions in structure in the immediate neighborhood of the site.

Another possible contribution to the ΔV can come from release of a bound sodium ion simultaneously with ethidium binding, since hydration of ions is normally accompanied by a decrease in volume (Neuman et al., 1973). In this case replacement of sodium by lithium in the buffer should result in a change in ΔV due to the difference in hydration of the two ions. The ΔV's for the two buffer systems, however, are identical within the limits of experimental error, indicating that such ion release does not conspicuously contribute to ΔV.

The preceding observations can be related to the concept of hard and soft sites distinguished by Torgerson et al. (1979) on the basis of model studies. Hard sites are rigid and expel the ligand as pressure is raised, while soft sites are deformable and result in stabilized binding with increased pressure. The tRNA–ethidium system is therefore an example of a soft site, and we would conclude that when positively charged aromatics are intercalated into nucleic acid stacked structures the decrease in volume is considerably greater than that due to simple stacking of aromatic rings, and binding is readily stabilized by pressure. Although the present study refers to transfer RNA, the previous considerations indicate that qualitatively similar effects should be expected upon intercalation of ethidium in DNA and other nucleic acids.

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In Vitro Ribosomal Ribonucleoprotein Transport upon Nuclear Expansion†

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ABSTRACT: The interdependence of nuclear rRNA release and nuclear size is investigated in macronuclei isolated from *Tetrahymena*. Nuclei are induced to contract and to expand, without any structural disintegration of the nuclear envelope, by final Ca^{2+}/Mg^{2+} (3:2) concentrations of 5 and 1.5 mM, respectively. Upon expansion, the average volume of nuclei increases from 600 ± 42 to 811 ± 76 μm^3. Concomitantly, nuclei begin to release RNA following saturation kinetics. This RNA release stops immediately upon nuclear contraction. Similar to the in vivo situation, only advanced rRNA processing products are released in the form of ribosomal precursor particles, as identified in detail by polyacrylamide gel electrophoresis and rate zonal and isopycnic density gradient centrifugation. Three particle types are released having average buoyant densities of 1.495, 1.470, and 1.532 g/cm^3, exhibiting average sedimentation coefficients of 62, 62, and 35 S, and containing the immediate precursor to the 26S rRNA, 26S rRNA, and 17S rRNA, respectively. The rRNP release is ATP independent and noncoincident with the release of endogenous nuclear P_i, though it is Be^{2+} sensitive. Our data are compatible with the views that nuclear expansion is the prerequisite rather than the cause for the rRNP release and that nuclear pore complex associated ATPases play only, if at all, a minor role in nucleocytoplasmic exchange of rRNP.

The transport of RNA from the nucleus to cytoplasm presumably is an important multiregulated process in gene expression of eukaryotic cells. An intrinsic role herein is often ascribed to the nuclear envelope, especially to its pore complexes which represent the only direct contact sites and the major, if not exclusive, passageways for RNA between the nucleus and cytoplasm [for reviews see, e.g., Wunderlich et al. (1976), Fry (1977), and Harris (1978)]. Circumstantial evidence is often interpreted as to indicate an active, i.e., ATP

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