D., Eds.) pp 493-516, Academic Press, New York, San Francisco, and London.
Knowles, J. R. (1972) *Acc. Chem. Res. 5*, 155-160.
Le Pecq, J.-B. (1973) in *Fluorescence Techniques in Cell Biology* (Thaer, A. A., & Sernetz, M., Eds.) pp 301-310, Springer-Verlag, Berlin, Heidelberg, and New York.
Liebman, M., Rubin, J., & Sundaralingham, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4821-4825.
Lochman, E.-R., & Micheler, A. (1979) *Photochem. Photobiol. 29*, 1199-1204.
Maly, P., Rinke, J., Ulmer, E., Zwieb, C., & Brimacombe, R. (1980) *Biochemistry 19*, 4179-4188.
Morgan, J., & Brimacombe, R. (1973) *Eur. J. Biochem. 37*, 472-480.
Morgan, A. R., Lee, J. S., Pulleyblank, D. E., Murray, N. L., & Evans, D. H. (1979a) *Nucleic Acids Res. 7*, 547-569.
Morgan, A. R., Evans, D. H., Lee, J. S., & Pulleyblank, D. E. (1979b) *Nucleic Acids Res. 7*, 571-594.
Singh, H., & Vadasz, J. A. (1978) *Photochem. Photobiol. 28*, 539-545.
Song, P.-S., & Tapley, K. J. (1979) *Photochem. Photobiol. 29*, 1177-1197.
Tao, T., Nelson, J. H., & Cantor, C. R. (1970) *Biochemistry 9*, 3514-3522.
Ulmer, E., Meinke, M., Ross, A., Fink, E., & Brimacombe, R. (1978) *Mol. Gen. Genet. 160*, 183-193.
Urbanke, C., Römer, R., & Maass, G. (1973) *Eur. J. Biochem. 33*, 511-523.
Volckaert, G., & Fiers, W. (1977) *Anal. Biochem. 83*, 228-239.
Zwieb, C., & Brimacombe, R. (1980) *Nucleic Acids Res. 8*, 2397-2411.

Dynamic Structure of DNA Complexes. Fluorometric Measurement of Hydrogen–Deuterium Exchange Kinetics of DNA-Bound Ethidium Dimer and Acridine–Ethidium Dimer†

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ABSTRACT: The hydrogen–deuterium (H–D) exchange kinetics of free and DNA-bound ethidium dimer and acridine–ethidium heterodimer were measured by stopped flow using fluorescence detection. This technique allowed a very accurate measurement of the exchange process. The H–D exchange kinetics were measured in various environments. In some cases, it was observed that the H–D exchange was much faster than the dissociation rate of dimer–DNA complexes. This showed that the exchange was taking place directly from the bound state. Furthermore, the action of a catalyst (imidazolium ion) on the rate of H–D exchange showed that a dynamic structural fluctuation of the ligand in its DNA complex was a necessary step on the exchange process.

Measurements of hydrogen exchange kinetics have furnished important information on the dynamic structure of proteins and nucleic acids [review by Woodward & Hilton (1979)]. Most of the data have been obtained by measuring tritium exchange. Recently, hydrogen exchange in nucleic acids has been studied by a new approach that takes advantage of the nucleic acid UV absorption change upon deuteration (Mandal et al., 1979).

Schreier & Baldwin (1976) demonstrated the usefulness of hydrogen exchange measurements in the study of the interaction kinetics of ligands with macromolecules. They showed that under some conditions, very small dissociation rate constants can be measured. Along this line, Mandal et al. (1980) measured the hydrogen–deuterium exchange of ethidium bromide in its complex with DNA using absorption spectroscopy. They have shown in their study the potentiality of this method to determine the binding parameters of a dye–DNA complex.

Furthermore, it is well-known that the fluorescence quantum yields of several dyes, including ethidium bromide, are from 2 to 4 times higher in D_2O than in H_2O (Stryer, 1966; Olmsted & Kearns, 1977). The kinetics of hydrogen–deuterium exchange of fluorescent compounds could therefore, in some cases, be very easily measured by fluorescence spectroscopy, as examplified by the work of Nakanishi et al. (1980) on tyrosine and tryptophan.

Recently, we have described the DNA binding properties of several bifunctional intercalators (Le Pecq et al., 1975, Roques et al., 1976; Delbarre et al., 1977; Gaugain et al., 1978a,b; Capelle et al., 1979; Markovits et al., 1979; Roques et al., 1979; Pelaprat et al., 1980; Markovits et al., 1981a,b; Delbarre et al., 1981; Reinhardt et al., 1982). We have shown that these molecules bind to DNA with a very high binding constant, which could be as high as 10^{10} M^{-1} in a physiological salt environment. Some of these molecules elicit strong antitumor properties (Roques et al., 1979; Pelaprat et al., 1980). Ethidium dimer (EthDi) and acridine–ethidium dimer (AcEthDi) are molecules that could represent interesting models for H–D exchange studies. Their fluorescence is

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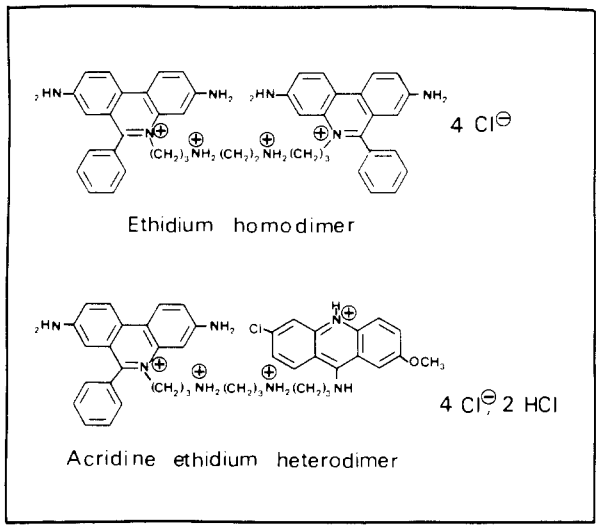

FIGURE 1: Chemical structures of ethidium dimer (EthDi) and ethidium–acridine dimer (AcEthDi).

strongly enhanced upon binding to DNA, and the AcEthDi fluorescence is base-sequence dependent (Gaugain et al., 1978b). In addition, their fluorescence is significantly enhanced in D_2O. The dissociation rates of these DNA–dimer complexes are small and difficult to measure directly. Therefore, measuring H–D exchange kinetics in DNA–dye complexes could represent an interesting way to determine the dissociation rate constants of these DNA complexes. A fluorometric study of H–D exchange kinetics of ethidium dimer and acridine–ethidium dimer in their complex with DNA was therefore undertaken.

As it will be shown in this paper, the rates of H–D exchange for these dimers in their DNA complexes are very accurately measured with stopped-flow techniques, using fluorescence detection. From experiments that will be presented and discussed in this paper, it is shown that the dissociation rate of DNA–dimer complexes is almost never a limiting step in H–D exchange and that the rate of the dynamic structural fluctuations of the DNA–dimer complex actually limits this exchange.

Experimental Procedures

Materials

The synthesis and purification of ethidium dimer (EthDi) and acridine–ethidium dimer (AcEthDi) were described by Gaugain et al. (1978a). The chemical structures of these compounds are shown in Figure 1. Ethidium bromide was purchased from Sigma.

Calf thymus DNA (Boehringer) was sonicated under nitrogen at 4 °C as described previously (Saucier et al., 1971; Le Pecq et al., 1975). The sedimentation coefficient of sonicated DNA measured by analytical ultracentrifugation was 6–7S at 20 °C. *Micrococcus luteus* DNA (Sigma) was purified by three phenol extractions. Poly[d(A-T)]·poly[d(A-T)] (Boehringer) was used without further purification.

Molar DNA–nucleotide concentrations were determined by using the following extinction coefficients at 260 nm: calf thymus, 6400 M^{-1} cm^{-1}, and *M. luteus*, 6900 M^{-1} cm^{-1} (Muller & Crothers, 1975); poly[d(A-T)]·poly[d(A-T)], 6800 M^{-1} cm^{-1} (Wells et al., 1970). Dye concentrations were measured according to the following extinction coefficients: $\epsilon_{490} = 1.212 \times 10^4$ M^{-1} cm^{-1} and $\epsilon_{450} = 7.908 \times 10^3$ M^{-1} cm^{-1}, respectively, for ethidium dimer and acridine–ethidium dimer.

Deuterium oxide (99.8%) was purchased from the Commissariat à l'Energie Atomique (Saclay, France). All other chemicals were of the best grade commercially available.

Methods

Fluorescence Measurements. Measurements were made in a SLM 800 spectrofluorometer (Urbana, IL) with a double monochromator for excitation and a single monochromator for emission and equipped with a thermostated cell holder. This instrument was associated with a 9315 Ortec photon counter, interfaced to a Minc Digital computer through a 779 Ortec interface controller.

Stopped-Flow Measurement of Hydrogen–Deuterium Exchange of Ethidium Dimer and of Acridine–Ethidium Dimer. A Durrum Gibson D110 stopped-flow instrument equipped with fluorescence detection was used. It was interfaced to a Minc Digital computer through a Digital MNCAA analogic Digital converter (12 bits). The maximum acquisition frequency was 10^4 Hz. One-thousand experimental points per relaxation were stored under computer control. The number of experimental points was further reduced to 200 by sequentially averaging points by groups of five. Through program control, variable sequential frequencies could be selected, and several experimental determinations could be accumulated and/or subtracted.

The mixing dead time of the instrument was found to be less than 2.5 ms. The slit width of the excitation monochromator (530 nm) was set at 2 mm. Fluorescence emission was observed through Corning C.S. 3–69 and Kodak Wratten No. 24 filters. The instrument was thermostated at 22 °C.

The dye–DNA complex solutions were made in H_2O buffer and equilibrated for 16 h before use. Sonicated calf thymus DNA was used to prevent the formation of ternary complexes (Capelle et al., 1979). These dye–DNA solutions were mixed in the stopped-flow instrument with an equal volume of the same buffer in D_2O.

Under standard conditions, calf thymus DNA was largely in excess (10^{-4} M in base pairs) over the dye (2×10^{-6} M). As these concentrations were much higher than the dissociation constant of the dimer (5×10^{-9} M), no dissociation of the complex was expected upon dilution. Nevertheless, control experiments were performed to verify that no relaxation due to the complex dissociation took place.

A few experiments were performed by mixing the DNA–dye complex in D_2O buffer with H_2O buffer. It was then verified that the observed signal was exactly symetrical to that observed under the standard conditions (DNA–dye complex in H_2O diluted with D_2O).

Computations. Data stored in the Minc computer were analyzed by nonlinear regression by using the Marquardt algorithm for a single- or double-exponential process (Marquardt, 1963). A Fortran program was kindly provided by Dr. Rigler and Dr. Nilsson from the Karolinska Institut (Stockholm). To decide whether a double-exponential process statistically improved the fitting of the data, an F test was used according to Bevington (1969).

Binding Parameters of Dimers. On rate constants for both dyes were directly measured in the stopped-flow apparatus by using pseudo-first-order analysis, as already described (Capelle et al., 1979). To measure the off rates of each dye, two different procedures had to be used:

(*i*) *Acridine–Ethidium Dimer.* The dissociation rate was deduced from the exchange kinetics of the dye between *M. luteus* DNA and poly[d(A-T)]·poly[d(A-T)]. For this dye, fluorescence is dependent on DNA base composition when excited in the acridine band (λ_{ex} = 465 nm) (Gaugain et al., 1978b). The dye was first preincubated with *M. luteus* DNA for 16 h, in buffers at different ionic strengths, and then, the

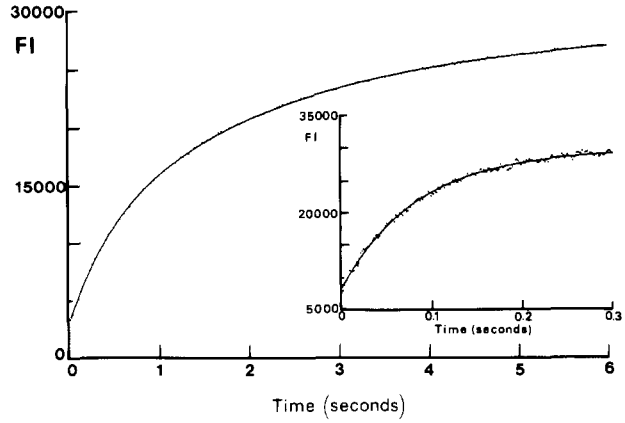

FIGURE 2: An example of hydrogen–deuterium exchange of EthDi–DNA complex and free EthDi (inset) observed by fluorescence after stopped-flow mixing. The dots represent the experimental data and the continuous lines the best computed fit. Buffer was 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)–0.1 M NaCl, pH 8.2. Temperature was 22 °C.

equilibrium was perturbed by the addition of an excess (40-fold) of poly[d(A-T)]·poly[d(A-T)]. As already shown, under these conditions, the observed relaxation rate is identical with the dissociation rate of the complex (Capelle et al., 1979). In the case of a short relaxation time ($\tau \leq 100$ s), the exchange kinetics were followed in the stopped-flow apparatus. In the other case, the variation of the fluorescence was measured in the SLM 800 fluorometer. In both cases, data were stored and analyzed under computer control, as described above.

(*ii*) *Ethidium Dimer.* In this case, the dissociation rate could not be directly measured. An apparent dissociation rate was deduced from the on rate constant k_1, measured as described above, and from the binding equilibrium constant K_a, assuming that the following simple equation applies:

$$k_{-1} = k_1/K_a$$

K_a was measured according to Gaugain et al. (1978b).

Results

Fluorescence Measurements of Amino Proton Exchange. The fluorescence quantum yield of free ethidium bromide is enhanced 3.5-fold upon going from H_2O to D_2O (Olmsted & Kearns, 1977). Similarly, free ethidium dimer, DNA-bound ethidium dimer, free acridine–ethidium dimer, and DNA-bound acridine–ethidium dimer fluorescence quantum yields increase 2.9-, 2.1-, 2.2-, and 2.3-fold, respectively on going from H_2O to D_2O. Therefore, the kinetics of H–D exchange of both free and DNA-bound dimer could be followed with high accuracy by the stopped-flow technique with fluorescence detection. In Figure 2, typical kinetic exchange curves for free and bound EthDi are shown. The free-dimer kinetic exchange can be fitted with a single exponential. In contrast, two exponentials are necessary to fit the DNA dimer H–D exchange curve, as shown in Figure 2.

Statistical analysis of these data shows that both time constants and their corresponding amplitudes can be estimated in this particular case, for a 95% confidence limit, with an accuracy of ±4%. However, at this point, it must be underlined that the determination of the shortest relaxation time, and its corresponding amplitude, becomes much less accurate under conditions where exchange accelerates and comes close to the dead time of the stopped-flow instrument.

Schreier & Baldwin (1976) and Mandal et al. (1980) have shown that hydrogen–deuterium exchange of ligand–macromolecule interactions could be analyzed with the following scheme:

$$\text{DNA–L}_D \xleftarrow{k_b} \text{DNA–L}_H \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{DNA} + \text{L}_H \xrightarrow{k_f} \text{DNA} + \text{L}_D$$

The notations of Mandal et al. (1980) are used here, where k_b is the exchange rate of dimer hydrogens in the complex, k_f is the exchange rate of dimer hydrogens in the free state, k_{-1} is the off rate constant, and k_1 is the on rate constant.

Mandal et al. (1980) interpreted their stopped-flow absorption H–D exchange kinetic results using the steady-state approximation of Schreier & Baldwin (1976), which gives, for the exchange a single time constant

$$k_{ex} = \frac{k_{-1}k_f + k_b(k_1[\text{DNA}] + k_f)}{k_{-1} + k_1[\text{DNA}] + k_f} \quad (1)$$

On the other hand, it must be noticed that when the H–D exchange is measured by absorption, both free and bound ligand contribute to the signal, according to their respective concentration and molecular extinction coefficients. Generally, the molecular extinction coefficients of bound and free dyes are not very different.

When fluorescence detection is used, contribution to the exchange of either the free or bound dye may become predominant because large differences can occur between the quantum yield of fluorescence bound and free dyes. It is therefore important to compute each species contribution to the fluorescence change separately and determine precisely when steady-state approximation could be applied. This calculation has been performed and is given in the supplementary material (see paragraph at end of paper regarding supplementary material).

This calculation shows that, because the DNA-bound dimer is much more fluorescent than the free dye and its DNA binding constant is high, the fluorescence variation resulting from the exchange can be accurately described by a single exponential, especially when DNA is in excess.

In this case, the corresponding time constant λ_2 (eq 9a in the supplementary material) is not significantly different from k_{ex} obtained by the steady-state approximation (eq 1) (Mandal et al., 1980) if $k_b \ll k_1[\text{DNA}]$ or $k_b \ll k_f$. Furthermore, in most cases studied here, $k_1[\text{DNA}] \gg k_{-1}$ and k_{ex} reduces to

$$k_{ex} = k_b + k_{-1}k_f/(k_f + k_1[\text{DNA}]) \quad (2)$$

In this situation, the rate of exchange is the sum of two terms:

$$k_{ex} = (k_{ex})_b + (k_{ex})_d \quad (3)$$

with

$$(k_{ex})_b = k_b \quad (4)$$

and

$$(k_{ex})_d = \frac{k_{-1}k_f}{k_f + k_1[\text{DNA}]} \quad (5)$$

The first one $(k_{ex})_b$ represents the direct exchange from the bound state. The second one $[(k_{ex})_d]$ takes into account the possibility of an indirect exchange via the free state, during the dissociation–reassociation kinetic process at equilibrium.

For the two ligand macromolecule complexes already studied (Schreier & Baldwin, 1976; Mandal et al., 1980), k_b was found negligible, and the binding parameters of the complexes could be evaluated.

To check whether such conditions could be met here, the variations of k_{ex} as a function of pH, DNA concentration, and ionic strength were measured. The effect of ionic strength on

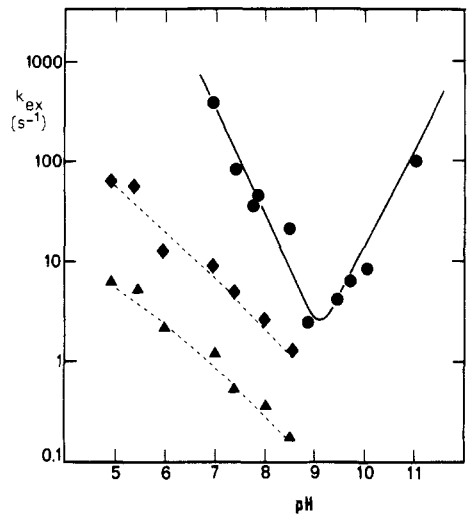

FIGURE 3: Effect of pH on H–D exchange rate. The rate constant for the H–D exchange of free EthDi (●) and the two rate constants for the H–D exchange of DNA-bound EthDi (▲, ♦) are shown as function of pH. All solutions contained 10^{-2} M NaCl and 10^{-3} M phosphate, 10^{-3} M cacodylate, or 10^{-3} M acetate buffer.

the exchange process is of special interest because, in the case of the dimers, the dissociation rate constant increases approximately as a function of the fourth power of the monovalent cation concentration (Gaugain et al., 1978b).

Effects of pH on H–D Exchange of Free and DNA-Bound Ethidium Dimer. The effect of pH on the H–D exchange of free ethidium dimer has been examined between pH 7 and 12 (Figure 3). In the case of free EthDi, if one plots the logarithm of the exchange rate constant against the pH, a typical V-shaped curve with a minimum located close to pH 9 is obtained. From this curve, it can be concluded that the protons can exchange via two different pathways, involving either H_3^+O or OH^- as a catalyst. The catalytic constants deduced from these data are 3×10^9 M^{-1} s^{-1} and 10^5 M^{-1} s^{-1} for H_3^+O and OH^-, respectively. It is interesting to note that similar values were obtained by Mandal et al. (1980), in the case of H–D exchange of the ethidium bromide amino proton.

For the DNA-bound EthDi, the study was only conducted between pH 5 and 9. Whereas a single relaxation is observed for free EthDi, two well-distinguished relaxations are observed for the DNA-bound dimer. The variations as a function of the pH of the two corresponding relaxation times are less pronounced than in the case of the free dye. Furthermore, it needs to be pointed out that DNA complexation of the dye causes a dramatic slow down in the exchange kinetics. For instance, at pH 7, the time constant for the exchange is increased almost 1000-fold on DNA complexation.

Effect of DNA Concentration on Rate of H–D Exchange of Ethidium Dimer and Ethidium–Acridine Heterodimer. Figure 4 shows the effect of DNA concentration on the H–D exchange kinetics measured by fluorescence for EthDi and AcEthDi. When the dye is in excess, the results are difficult to interpret since the steady-state approximation does not apply (as it is shown in the calculation presented in the supplementary material). Therefore, only those measurements obtained when DNA is in excess or the free dye concentration is negligible are presented.

Because of the known binding parameters of the two dimers (Gaugain et al., 1978a), the concentration of the free dimer can be neglected as soon as the ratio DNA-P/dye becomes larger than 4. Furthermore, it is known that the binding mode changes when DNA-P/dye becomes greater than 8. At that point, a small but significant change of the two time constants

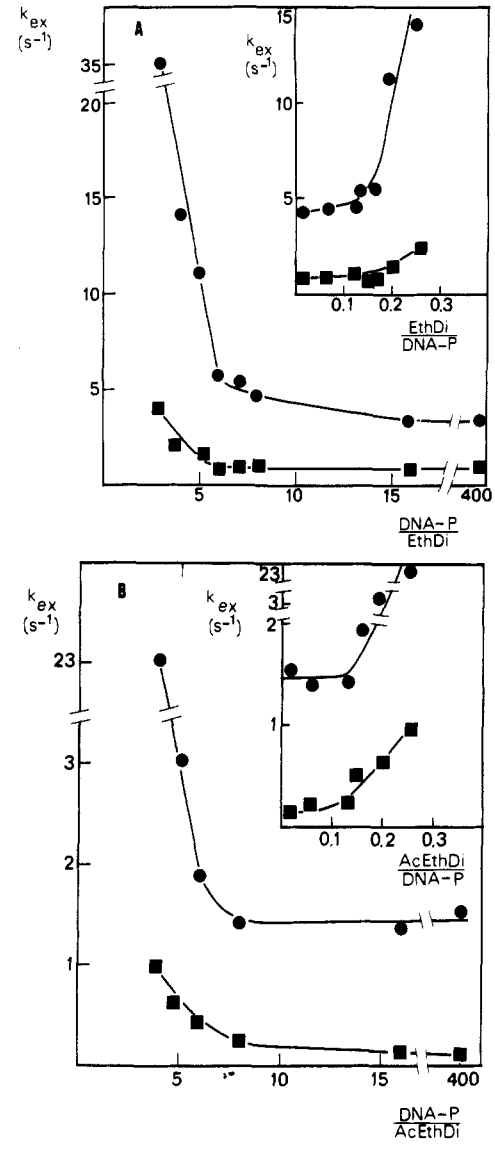

FIGURE 4: Variation of H–D exchange rate with the ratios DNA-P/dimer and dimer/DNA-P (inset): (A) ethidium dimer; (B) acridine–ethidium dimer. EthDi concentration was 10^{-6} M and AcEthDi concentration 2×10^{-6} M. These experiments were performed at 22 °C, pH 8, in solutions containing 10^{-2} M NaCl– 2×10^{-3} M Tris-HCl, pH 7.4.

is observed, whereas the two corresponding amplitudes remain constant.

Effect of Ionic Strength on Rate of Exchange of Ethidium Bromide, Ethidium Dimer, and Acridine–Ethidium Heterodimer. The equilibrium of an electrically charged ligand with a polyelectrolyte is strongly affected by the ionic strength of the medium. For positively charged ligand and a polyanionic macromolecule (Record et al., 1976), the logarithm of the binding constant decreases linearly with the logarithm of the monovalent cation concentration, with a slope proportional to the number of charges in interaction in the complex (1 for ethidium bromide; 4 for EthDi and AcEthDi).

In Figure 5, the measured values of the rate of exchange (k_{ex}) are shown as a function of sodium concentration. They are compared to the calculated contributions in the exchange of the dissociation–reassociation process $(k_{ex})_d = k_{-1}k_f/(k_f + k_1[DNA])$ as defined in equation 5.

Effect of a Çatalyst on Exchange Process. As will be discussed later, it became apparent that under some defined conditions (low ionic strength, high DNA concentration, etc.), the measured exchange rates were equal to k_b. As in the study

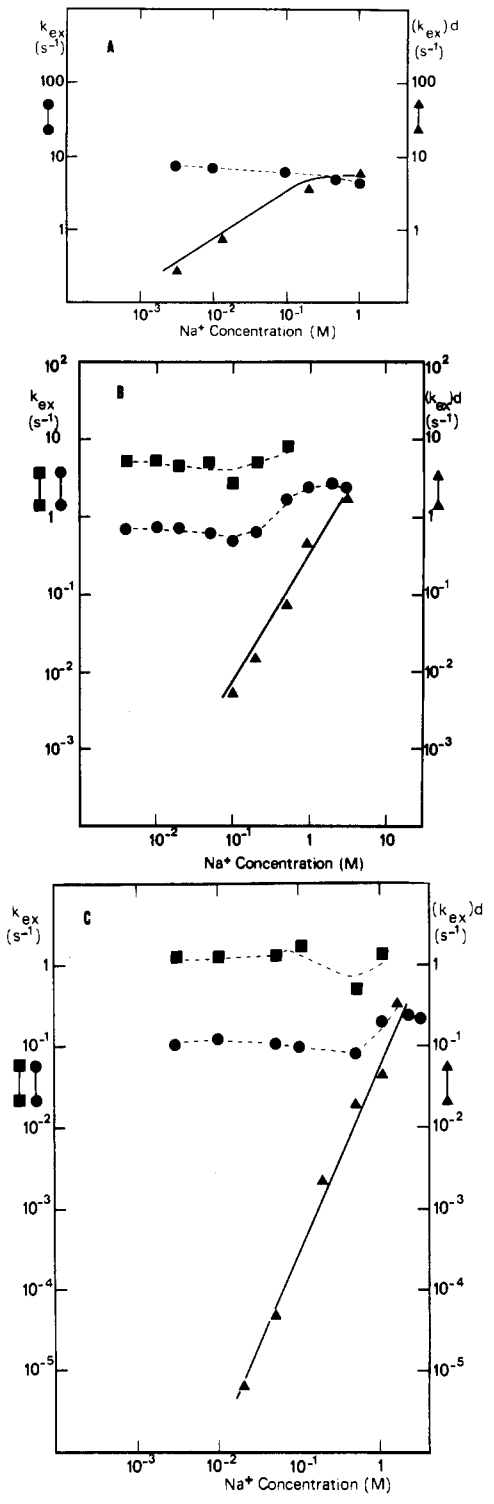

FIGURE 5: Comparison between observed ionic strength dependence of H–D exchange and computed contribution to exchange of dissociation–reassociation process [$(k_{ex})_d$, eq 5]. (A) Ethidium bromide–DNA complex. Observed rate constant (●) and computed $(k_{ex})_d$ (▲). (B) Ethidium dimer–DNA complex. (C) Acridine–ethidium dimer–DNA complex. For (B) and (C), the variation of the two observed rate constants are shown (●, ■), as well as the computed value of $(k_{ex})_d$ (▲). All measurements were done at 22 °C by adding the indicated concentration of NaCl. All solutions contained 2×10^{-3} M Tris-HCl, pH 7.4, 2×10^{-6} M dyes, and 2×10^{-4} M DNA-P.

of hydrogen exchange in nucleic acids (Mandal et al., 1979), a critical experiment to perform was to investigate the effect of catalysts on the exchange process. Two extreme behaviors can be expected: (i) The exchange increases linearly and continuously with the catalyst concentration. In that case, it would be concluded that the action of the catalyst results from a bimolecular process and its action is only hindered by limited accessibility of the ligand in the bound state. (ii) The exchange rate constant increases first with the catalyst concentration, later reaching a plateau at high catalyst concentration. Such behavior is typical of H–D exchange in nucleic acids and has been interpreted according to the following scheme (McConnell & Von Hippel, 1970; Mandal et al., 1979):

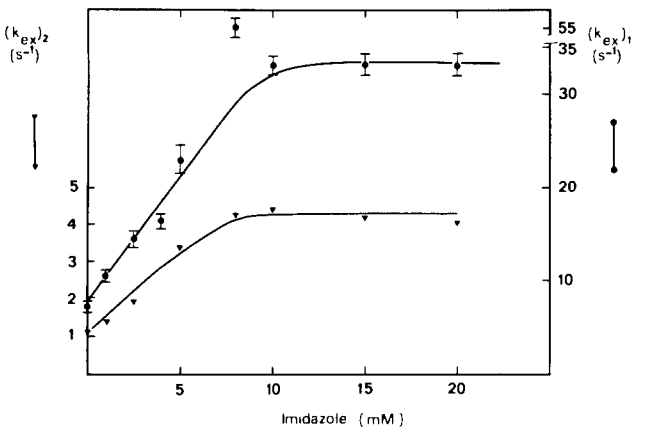

FIGURE 6: Imidazole catalysis of H–D exchange of ethidium dimer–DNA complex. Rates were determined at pH 7, at 22 °C. The total cation concentration was adjusted to 10^{-2} M by the addition of NaCl.

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_{ch}[C]} \text{exchanged} \qquad (6)$$

The exchange cannot occur when DNA is in a closed conformation (hydrogen bonded). It only occurs when hydrogen bonds break and DNA base pairs open. The opening of the base pairs is controlled by the rate of opening (k_{op}) and closing (k_{cl}). In the open state, the H–D exchange rate constant is $k_{ch}[C]$ ([C] being the catalyst concentration). It was shown (McConnell & Von Hippel, 1970) that

$$k_{ex} = k_{op}k_{ch}[C]/(k_{cl} + k_{ch}[C]) \qquad (7)$$

At high catalyst concentration, $k_{ex} \rightarrow k_{op}$, and at low catalyst concentration ($k_{cl} \gg k_{ch}[C]$)

$$k_{ex} \neq k_{op}k_{ch}[C]/k_{cl} \qquad (8)$$

Here, imidazole was found to be the most efficient catalyst for the H–D exchange of DNA-bound EthDi. Because catalytic efficiency was found to decrease with pH, the protonated form was thought to be the actual catalyst. At pH 8, the catalytic constant for free EthDi exchange is found equal to 5.6×10^5 M^{-1} s^{-1}. The variation of the H–D exchange kinetics of DNA-bound EthDi as a function of imidazole concentration is shown in Figure 6.

It is clearly observed that both time constants level off at high catalyst concentrations while relative amplitudes remain constant. In the case of AcEthDi, behavior is qualitatively the same, but the leveling off of the rate constants is obtained at much lower catalyst concentrations (results not shown).

Discussion

Hydrogen exchange kinetics have been widely used to measure dynamic fluctuations in macromolecules and more recently to estimate the dissociation kinetics of very stable ligand macromolecule complexes. Because deuterium exchange induces a small variation of molecular extinction coefficients of some chromophores (a few percent), UV absorption spectroscopy and stopped-flow have recently been used

to study the hydrogen exchange process in nucleic acids (Mandal et al., 1979).

In this paper, the stopped-flow technique associated with fluorescence detection was used to study hydrogen exchange of DNA–dye complexes. The fluorescence quantum yield of somee molecules upon deuterium exchange can increase by a factor as large as 3. It follows that the sensitivity and the accuracy of the hydrogen exchange kinetic measurement can then become extremely high, as demonstrated in this work. At this point, it is worth recalling the mechanism of molecular fluorescence variation upon deuteration. For some compounds, it has been shown that the variation results from a difference in the kinetics of proton and deuteron release during the ionization process of the molecule in its excited state (Stryer, 1966). It follows that the fluorescence signal upon deuterium exchange may, in most cases, reflect the involvement of a single proton, corresponding to the group deprotonated in the excited state. In the case of ethidium, the mechanism of fluorescence enhancement by D_2O has been studied by Olmsted & Kearns (1977). They have demonstrated that the fluorescence enhancement is directly related to a difference between the rate of proton and deuteron transfer to solvent in the excited state of the molecule.

Therefore, it is clear that the fluorescence signal upon deuterium exchange involves the properties of the dye excited states. But, it is not clear whether one or both amino groups of ethidium are involved in this process.

A single relaxation is observed in the H–D exchange of free ethidium, free ethidium homodimer, and free acridine–ethidium heterodimer. As a single relaxation is still observed for the H–D exchange of DNA-bound ethidium, two relaxation processes are observed for the H–D exchange of the DNA-bound ethidium homodimer and DNA-bound acridine–ethidium heterodimer. Furthermore, for either dimer, the two relaxation times vary in parallel while experimental conditions are modified (pH, ionic strength, catalyst concentration, etc.), whereas the corresponding relative amplitudes remain constant. Ethidium homodimer, as well as ethidium–acridine heterodimer, is known to bind to DNA according to two different modes. In the first one, the dye covers four base pairs. This binding process is predominant for low values of the ratio dye/DNA-P. In the second one, the dye covers only two base pairs and behaves as a purely monointercalating agent. This later binding process is predominant when the ratio dye/DNA-P (r) becomes larger than 0.2 (Gaugain et al., 1978b). When this ratio (r) is varied from 0 to 0.25, the relative amplitude of the two relaxation times remains constant. A small increase in both relaxation times only is observed when r becomes greater than 0.12 (Figure 4). Therefore, the existence of the two relaxation processes cannot be correlated with a known heterogeneity in the DNA binding mode. The two relaxation processes probably reflect different kinds of DNA binding heterogeneity that have not been detected in the previous DNA binding study (Figure 4).

Two different pathways can potentially be followed for the hydrogen exchange of a ligand bound to a macromolecule (see scheme). One represents the direct hydrogen exchange from the bound state; the other corresponds to the hydrogen exchange via the free state and is controlled by the dissocation reassociation kinetics process.

Two extreme cases can therefore be considered: (i) The dissociation rate of the complex (k_{-1}) is much larger than the hydrogen exchange rate via the bound state ($k_b \ll k_{-1}$). In this situation, k_{ex} is given by eq 5, and k_{-1} can be deduced from hydrogen exchange measurement. (ii) $k_b \gg k_{-1}$. In this situation, eq 2 reduces to $k_{ex} = k_b$. In the system studied by Schreier & Baldwin (1976), the first situation is encountered, and the dissociation rate constant can be deduced.

In the systems studied here, the two different situations have been observed depending on the experimental conditions, as shown in Figure 5, where k_{ex} is compared to the calculated contribution of the exchange via the free state $(k_{ex})_d$ (eq 5). From these results, it is clear that at high ionic strength (k_{-1} large) the first situation applies. In contrast, at low ionic strength, the dissociation rate constant of the dimer is decreased dramatically; hence, the second case obviously applies; k_{ex} becomes much larger than $(k_{ex})_d$ and remains constant. Therefore, in this situation, $k_{ex} = k_b$. $(k_{ex})_d$ can also be made very small (eq 5) by increasing the free DNA concentration. In this case, k_{ex} remains constant while the DNA concentration increases (Figure 4). In the case of ethidium dimer, it is worth mentioning that at high ionic strength, when the H–D exchange is controlled by the complex dissociation process, a single relaxation for the H–D exchange was observed as expected.

At this stage, an important point to be examined was to determine whether the exchange in the bound state was controlled by the limited accessibility of the amino exchangeable groups or by a dynamic fluctuation at the level of the DNA–ligand complex. In order to distinguish between these two possibilities, the effect of catalysts on the exchange rates was a critical experiment. As already pointed out under Results, our results strongly favored the existence of a dynamic structural fluctuation of the DNA–dye complex. Here, the arguments were identical with those in favor of a dynamic fluctuation of the DNA structure (Teitelbaum & Englander, 1975a,b). Interestingly enough, the rate of H–D exchange at high catalyst concentration was within the same time range as the corresponding rate measured for proton exchange in ligand-free DNA. This suggested that a similar fluctuation mode (the opening of base pairs) governed the H–D exchange of the dye protons in the complex (Mandal et al., 1979).

Conclusions

This work demonstrates the usefullness of fluorescence detection in following deuterium–hydrogen exchange. Furthermore, the measurement of deuterium–hydrogen exchange of ethidium dimer and ethidium–acridine heterodimer bound to DNA gives evidence for a dynamic structural fluctuation of a ligand complexed with a macromolecule.

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Supplementary Material Available

Solution of differential equations applying to the hydrogen exchange kinetics of a ligand interacting with a macromolecule without making the steady-state approximation (6 pages). Ordering information is given on any current masthead page.

Registry No. Poly[d(A-T)], 26966-61-0; imidazole, 288-32-4.

References

Bevington, Ph. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 195–203, McGraw-Hill, New York.

Capelle, N., Barbet, J., Dessen, Ph., Blanquet, S., Roques, B. P., & Le Pecq, J. B. (1979) *Biochemistry 15*, 3354–3362.

Delbarre, A., Roques, B. P., & Le Pecq, J. B. (1977) *C. R. Hebd. Seances Acad. Sci., Ser. D 284*, 81–84.
Delbarre, A., Gaugain, B., Markovits, J., Vilar, A., Le Pecq, J. B., & Roques, B. P. (1981) in *Intermolecular Forces* (Pullman, B., Ed.) pp 273–283, D. Reidel Publishing Co., Dordrecht, Holland.
Gaugain, B., Barbet, J., Oberlin, R., Roques, B. P., & Le Pecq, J. B. (1978a) *Biochemistry 17*, 5071–5077.
Gaugain, B., Barbet, J., Capelle, N., Roques, B. P., & Le Pecq, J. B. (1978b) *Biochemistry 17*, 5078–5088.
Le Pecq, J. B., Le Bret, M., Barbet, J., & Roques, B. P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5078–5082.
Mandal, C., Kallenbach, N. R., & Englander, S. W. (1979) *J. Mol. Biol. 135*, 391–411.
Mandal, C., Englander, S. W., & Kallenbach, N. R. (1980) *Biochemistry 19*, 5819–5825.
Markovits, J., Roques, B. P., & Le Pecq, J. B. (1979) *Anal. Biochem. 94*, 259–264.
Markovits, J., Blanquet, S., Dessen, P., Roques, B. P., & Le Pecq, J. B. (1981a) *Biochem. Pharmacol. 30*, 1557–1562.
Markovits, J., Gaugain, B., Roques, B. P., & Le Pecq, J. B. (1981b) in *Intermolecular Forces* (Pullman, B., Ed.) pp 285–298, D. Reidel Publishing Co., Dordrecht, Holland.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.
McConnell, B., & Von Hippel, P. H. (1970) *J. Mol. Biol. 50*, 297–316.
Muller, W., & Crothers, D. M. (1975) *Eur. J. Biochem. 54*, 267–277.
Nakanishi, M., Kobayashi, M., Tsuboi, M., Takasaki, C., & Tamiya, N. (1980) *Biochemistry 19*, 3204–3208.
Olmsted, J., & Kearns, D. R. (1977) *Biochemistry 16*, 3647–3654.
Pelaprat, D., Delbarre, A., Le Guen, I., Le Pecq, J. P., & Roques, B. P. (1980) *J. Med. Chem. 23*, 1336–1342.
Record, M. T., Jr., Lohman, T. M., & De Hasseth, P. (1976) *J. Mol. Biol. 107*, 145–158.
Reinhardt, C., Roques, B. P., & Le Pecq, J. B. (1982) *Biochem. Biophys. Res. Commun. 104*, 1376–1385.
Roques, B. P., Barbet, J., Oberlin, R., & Le Pecq, J. B. (1976) *C. R. Hebd. Seances Acad. Sci., Ser. D 283*, 1365–1368.
Roques, B. P., Pelaprat, D., Le Guen, I., Porcher, G., Gosse, Ch., & Le Pecq, J. B. (1979) *Biochem. Pharmacol. 28*, 1811–1815.
Saucier, J. M., Festy, B., & Le Pecq, J. B. (1971) *Biochimie 53*, 973–980.
Schreier, A. A., & Baldwin, R. L. (1976) *J. Mol. Biol. 105*, 409–426.
Stryer, L. (1966) *J. Am. Chem. Soc. 88*, 5708–5712.
Teitelbaum, H., & Englander, S. W. (1975a) *J. Mol. Biol. 92*, 55–78.
Teitelbaum, H., & Englander, S. W. (1975b) *J. Mol. Biol. 92*, 79–92.
Wells, R. D., Larson, J. E., Grand, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465–497.
Woodward, C. K., & Hilton, B. D. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 99–127.

Characterization of a Complementary Deoxyribonucleic Acid Coding for the α Chain of Human Fibrinogen†

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ABSTRACT: A human liver cDNA library was screened for the α chain of fibrinogen with a cDNA clone from the corresponding bovine molecule as a hybridization probe. Several human clones coding for the α chain were identified, and one of these was used to rescreen the entire cDNA library of 18 000 recombinants. Plasmids with the largest cDNAs were isolated, and their inserts were sequenced. The largest cDNA insert contained 2224 base pairs, including a noncoding region at the 5′-end followed by a region coding for a signal peptide of 19 (or 16) amino acids and a mature protein of 625 amino acids, a stop codon of TAG, another noncoding region, and a poly(A) tail at the 3′-end. Eight tandem repeats of 39 base pairs were observed starting with nucleotide 905 (amino acid residue 270) and ending with nucleotide 1213 (amino acid residue 372). The identity in the nucleotide sequence in the tandem repeats ranged from 72 to 95% when compared to a consensus sequence. The predicted amino acid sequence for the mature polypeptide chain was 15 amino acids longer at the carboxyl-terminal end than that of the α chain isolated and sequenced from plasma fibrinogen. This indicates that minor proteolysis has taken place on the carboxyl-terminal end of the α chains, and this modification has probably occurred during secretion or circulation of the protein in plasma.

Fibrinogen[1] (M_r 340 000) is a plasma protein that participates in the final phase of blood coagulation (Marder et al., 1982). Each molecule consists of two sets of three different polypeptide chains designated α, β and γ, with molecular weights of 66 000, 52 000, and 46 500, respectively (McKee et al., 1966). Fibrinogen is a glycoprotein containing four carbohydrate chains, including one on each of the β chains and one on each of the γ chains (Iwanaga et al., 1968; Töpfer-Petersen et al., 1976). The α chain of fibrinogen is free of carbohydrate (Pizzo et al., 1972). The three pairs of chains in fibrinogen are held together by disulfide bonds and

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[1] The three chains in fibrinogen (factor I) have also been called Aα, Bβ, and γ (Blombäck, 1969). Plasmids were labeled as follows: p, plasmid; H, human; B, bovine; I, fibrinogen; α, α chain; 1, first plasmid identified.

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