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Relaxation Studies of Enzymes: Rapid Isomerization in Deoxyribonuclease I†

Stephen M. Feltch and John E. Stuehr*

ABSTRACT: Temperature-jump relaxation studies in deoxyribonuclease I were carried out at 10 °C and [I] = 0.1 M. The single observed relaxation time, which varied from 10^{-4} to 10^{-5} s, was characterized as a function of enzyme concentration, pH, and indicator concentration. The concentration and pH dependences of the relaxation time are in quantitative agreement with a mechanism involving an isomerization of the enzyme coupled to a rapid proton ionization process. The best fit forward and reverse isomerization rate constants are 6.5×10^3 and 7.2×10^4 s^{-1}, respectively; the apparent pK is 5.7. The addition of urea brought about reductions in both the amplitude of the relaxation effect and the enzyme activity.

Deoxyribonuclease I is an enzyme of molecular weight 31 000 that catalyzes the hydrolysis of native DNA to 5′-deoxyribonucleotides. Early work on the steady-state kinetics has done much to define the overall aspects of the reaction. The enzyme was found to be an endonuclease (Williams et al., 1961) having an absolute requirement for a divalent metal ion such as Mg^{2+} or Mn^{2+} (Wiberg, 1958). At least two phases of the reaction with native DNA have been recognized. The initial phase is characterized by both specificity (Weis et al., 1968) and the production of several single-stranded nicks before cleavage of the double strand (Dekker & Schachman, 1954). During the terminal phase there is little specificity (Laskowski, 1967) and autoretardation is observed (Vanecko & Laskowski, 1961). Both competitive product inhibition (Vanecko & Kaskowski, 1961) and decreasing affinity toward newly formed substrates (Vanecko & Laskowski, 1961; Cavalieri & Hatch, 1953) have been shown to be necessary to account for the latter phenomenon. Chemical modification experiments have implicated tyrosine (Hugli & Stein, 1971), serine (Poulos & Price, 1974), carboxyl (Poulos & Price, 1974), and histidine (Price et al., 1969) residues as essential to DNase activity. If indeed all of these groups are involved at the active site, the mechanism must be quite complex. To date, however, no detailed mechanistic scheme has been proposed. The required time sequence of events at the molecular level has not been characterized. We have therefore initiated a broad investigation to characterize as many rapid elementary steps as possible to help in the elucidation of the detailed mechanism. The principal investigative tool will be temperature-jump spectroscopy. The suitability of this technique for the study of such enzymatically important rapid processes as ionizations, configurational changes (isomerizations), association phenomena, enzyme–substrate binding, and enzyme–metal ion interactions is well documented (del Rosario & Hammes, 1970). This paper describes a rapid process involving the enzyme in the absence of substrates or cofactors.

Experimental Procedures

Reagents and Chemicals. Phosphocellulose was purchased from Accurate Chemical and Scientific Corp. Lima bean trypsin inhibitor and CNBr-activated Sepharose 4B were from Sigma Chemical Co. Bovine pancreatic deoxyribonuclease I was purchased from Sigma or Miles Laboratories. All other reagents were standard analytical grade. The indicators methyl red, bromthymol blue, and chlorophenol red were checked for purity by thin-layer chromatography, titration, and visible absorption spectra and found to be pure by these criteria. Urea was recrystallized from 70% ethanol.

Enzyme Purification. The crude enzyme was treated with phenylmethanesulfonyl fluoride to inactivate proteases. Typically, 1.0 g of protein was dissolved in 100 mL of 0.05 M Tris buffer, 5×10^{-3} M Ca^{2+}, pH 7.0, to which 10 mL of 0.02 M phenylmethanesulfonyl fluoride (in 95% ethanol) had been previously added. It was usually necessary to readjust the pH to 7.0 with 1.0 M base. The solution was allowed to stand at room temperature for 1 h, dialyzed vs. distilled water at 4 °C, and lyophilized. Some precipitation occurred after 1 h at room temperature and also after dialysis. This was removed by filtration through Whatman high-porosity fluted paper. Recovery of activity was >90%. The treated protein lost no activity after 24 h at room temperature, pH 7.0, in 0.05 M EDTA.

The phenylmethanesulfonyl fluoride treated enzyme was purified over a 2.5 × 70 cm column of phosphocellulose according to the procedure of Salnikow et al. (1970). The fractions containing enzyme activity were pooled, dialyzed vs. distilled water at 4 °C, and lyophilized. This material was further purified by affinity chromatography over a 2 × 25 cm lima bean trypsin inhibitor–agarose column according to the procedure of Otsuka & Price (1974). The purified DNase I was stored frozen in the buffer from the final column (0.02 M Tris and 5×10^{-3} M Ca^{2+}, pH 8.0) for up to several months without loss of activity.

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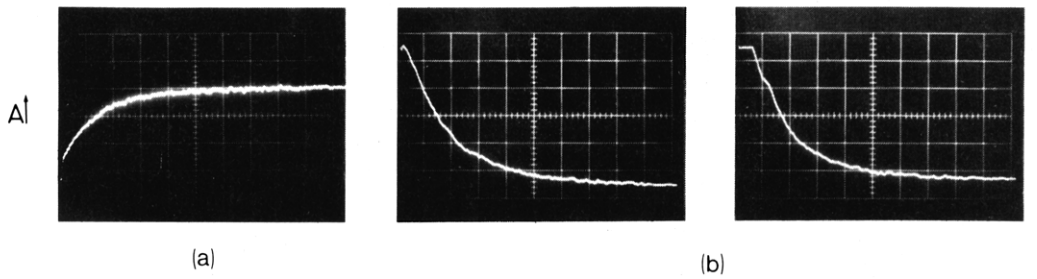

FIGURE 1: Typical relaxation photographs of DNase I at 10 °C. (a) DNase I concentration, 4 μM; methyl red, 190 μM; pH 6.94; 0.5-ms full scale. (b) DNase I concentration, 34 μM; bromthymol blue, 50 μM; pH 7.3; native enzyme (left), alkylated (right); 1-ms full scale.

The enzyme was of >95% purity on $NaDodSO_4$ gel electrophoresis (Weber & Osborn, 1969) and acrylamide gel electrophoresis[1] at pH 4.7 (Reisfield et al., 1963). The specific activity of the preparations ranged from 94 to 106 units/mg when assayed at pH 5.0 and 25 °C against a Mn-DNA substrate. This is in excellent agreement with previously reported values for purified DNase (Otsuka & Price, 1974).

DNase is composed of four isozymes (Salnikow et al., 1970), two of which differ in only one amino acid residue and the other two which differ only in the carbohydrate moiety. The specific activities of the isozymes are identical. Peptide maps of the four isozymes indicate no gross structural differences (Salnikow & Murphy, 1973; Liao, 1974). There was no difference between the kinetic behavior of the A isozyme as compared to a mixture of the four isozymes. We therefore routinely pooled all of the active fractions from the phosphocellulose column. The kinetic data presented under Results are for a mixture of all four isozymes.

Alkylation. The enzyme was alkylated by using iodoacetate in the presence of Cu^{2+} as described by Price et al. (1969). Under these conditions only a single histidine at the active site is alkylated. Excess reagents and metal ions were removed by dialysis.

Kinetic Runs and Data Treatment. Aliquots of the enzyme were thoroughly dialyzed at 4 °C against 0.05 M EDTA, followed by 0.1 M KNO_3 to remove storage buffer and metal ions. Appropriate amounts of indicator were added from 1 × 10^{-3} M stock solutions in 0.1 M KNO_3. The pH was adjusted at 10 °C on a Beckman Expandomatic pH meter with 0.1 M KOH or 0.1 M HNO_3. All kinetic runs were done at 10 °C on a temperature-jump spectrometer (Messanlagen Studiengesellschaft) employing at 6.4 °C temperature rise. The enzyme lost no activity after repeated jumps. Blanks of each of the indicators showed no relaxation effects in the time region under consideration.

Data from photographs of relaxation effects were first plotted on semilog paper to demonstrate linearity and then processed by a nonlinear least-squares treatment on a Univac 1108 computer to obtain the best values for the relaxation times. All data points were from an average of at least two photographs.

Results

A single relaxation time was observed for solutions of enzyme and indicator over the following range of experimental conditions: $[E^\circ]$ = 4–460 μM, $[In^0]$ = 10–190 μM, and pH 5.3–7.7. Half-times for the process varied from 11 to 92 μs. Photos of typical relaxation effects are shown in Figure 1. The dependence of the reciprocal relaxation time on enzyme concentration is given in Figure 2. A 100-fold change in $[E^0]$ produces a fivefold change in τ^{-1} with bromthymol blue as the pH indicator. With chlorophenol red only a 15-fold increase in $[E^0]$ produces the same effect on τ^{-1}. The pH dependence at constant enzyme concentration for various indicators is shown in Figure 3. As may be seen, the dependence at 25 μM DNase is quite different for the various indicators. Methyl red (pK_{In} = 5.06) has τ^{-1} values which go through a minimum at a pH of ~6.0. On the other hand, τ^{-1} values obtained with both chlorophenol red (pK_{In} = 6.05) and bromthymol blue (pK_{In} = 7.10) steadily decrease with increasing pH. However,

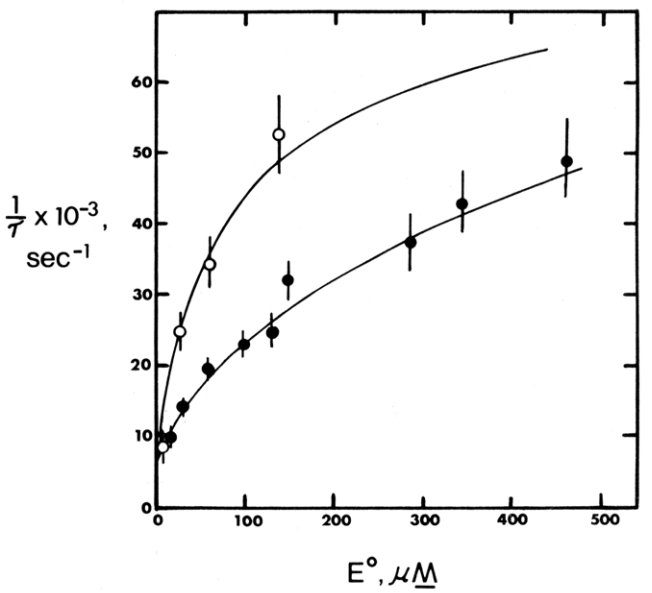

FIGURE 2: Dependence of $1/\tau$ upon overall enzyme concentration at 10 °C and pH 7.0. (○) Chlorophenol red, 30 μM; (●) bromthymol blue, 50 μM. Solid lines calculated with constants given in the text.

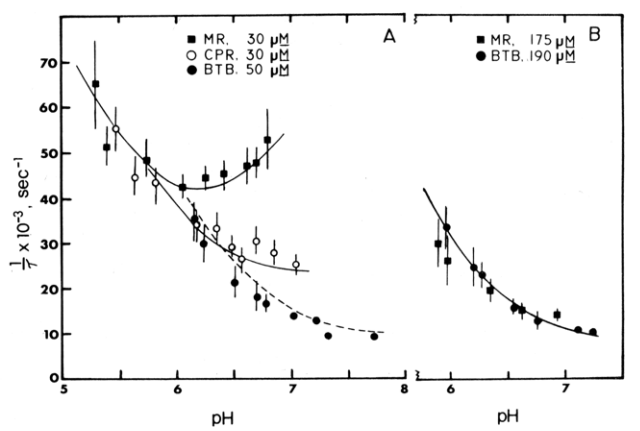

FIGURE 3: Dependence of $1/\tau$ upon pH at 10 °C for three indicators: methyl red (MR), chlorophenol red (CPR), and bromthymol blue (BTB). Concentrations are as indicated. (A) DNase concentration, 25 μM. (B) DNase concentration, 4 μM. Lines through data points were calculated with constants given in the text.

[1] The concentrations of the reagents in the buffers were slightly modified to produce pH 4.7 gels. Neutral red was used as a tracking dye.

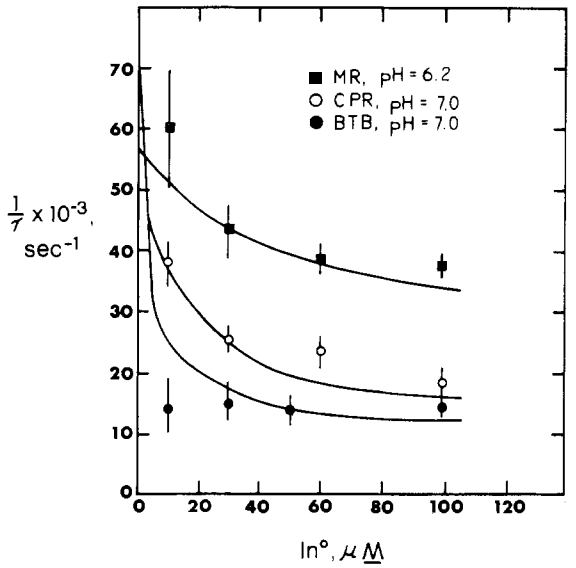

FIGURE 4: Dependence of $1/\tau$ upon overall indicator concentration for 25 μM DNase at 10 °C for methyl red (MR), chlorophenol red (CPR), and bromthymol blue (BTB). Solid lines are calculated with constants given in the text.

the chlorophenol red data level off at higher values of τ^{-1} as the pH increases. At 4 μM DNase with a 4× increase in the indicator concentration, τ^{-1} becomes independent of the indicator. Figure 4 shows the dependence of the process on indicator concentration.

To ensure that the effect was not due to an enzyme–indicator interaction, each indicator used was tested under our experimental conditions by several methods for evidence of binding to the enzyme. Addition of indicator to the assay mix did not affect the specific activity of the enzyme. No detectable binding was measured on equilibrium dialysis. Addition of enzyme to a solution of indicator did not perturb the visible spectrum of the indicator (i.e., no difference spectrum was seen). Also the *inverse* dependence of τ^{-1} on indicator concentration (Figure 4) *precludes* the appearance of the indicator in the rate-determining step.

Several mechanisms involving self-association of the enzyme were inconsistent with the data. These predict a quadratic dependence upon $[E^0]$. As a consequence, Figure 1 should show a τ^{-1} vs. $[E^0]$ plot in which the slope of the curve progressively increases as $[E^0]$ increases. This is not the case: the slope actually decreases as $[E^0]$ increases.

A simple proton transfer mechanism was also discounted, for three reasons. Treatment of the data via a computerized nonlinear least-squares procedure yielded values of association rate constants on the order of 10^{10} M^{-1} s^{-1}. While this is compatible with a diffusion-controlled process involving small molecules, it is too fast by about 2 orders of magnitude for a macromolecular species, Second, a single set of rate constants could not be found which simultaneously fit the pH and concentration dependences with even a single indicator. Finally, the constants derived with bromthymol blue as the indicator failed to predict the experimental observations with the other two indicators.

The pH and concentration dependences suggest a mechanism involving a term composed of the sum of the hydrogen ion concentration and an enzyme concentration. The simplest such mechanism—a proton transfer—was shown as described above not to fit the data. The curvature of the concentration dependence at constant pH further suggests that an enzyme concentration term appears in the denominator of the rate expression. To generate this type of dependence it is only

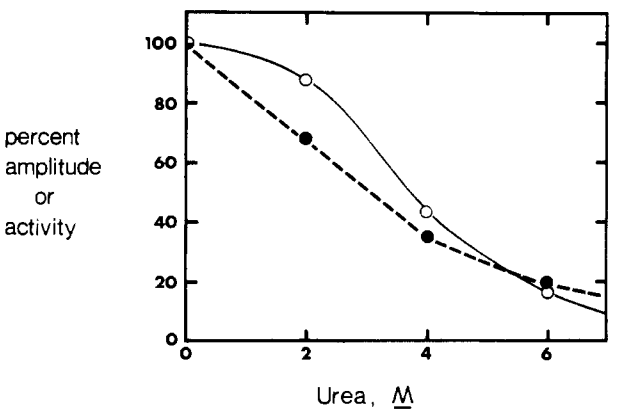

FIGURE 5: Variation of relative relaxation amplitude (●) at 10 °C and enzyme activity (○) (standard assay conditions; see text for details) upon concentration of urea; for amplitude measurements, $[E^0]$ = 25 μM, pH 7.0; indicator is bromthymol blue, 50 μM.

necessary to couple the proton transfer to a slower conformational change. Several such mechanisms involving single and multiple proton transfers as derived (Feltch & Stuehr, 1979) were tested. These were fitted to the data on a Univac 1108 computer via a nonlinear least-squares procedure.

The mechanism which best represented the data was a slow isomerization coupled to a single rapid proton transfer (Scheme I)

Scheme I

$$E'H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EH \overset{K_a}{\rightleftharpoons} E + H$$

$$HIn \overset{K_{In}}{\rightleftharpoons} H + In$$

$$H_2O \overset{K_w}{\rightleftharpoons} H^+ + OH^-$$

For this mechanism the reciprocal relaxation time is

$$\tau^{-1} = \frac{\dfrac{[E]}{1 + \alpha + \beta} + [H]}{\dfrac{[E]}{1 + \alpha + \beta} + [H] + K_a} k_r + k_f \qquad (1)$$

where $\alpha = [In]/(K_{In} + [H])$ and $\beta = [OH]/(K_w + [H])$. This is essentially the isomerization mechanism proposed by French & Hammes (1965) for ribonuclease. The relaxation time expression (eq 1) differs from theirs in that the terms involving free enzyme are retained.

The best computer-fitted constants were obtained by using initial estimates of K_a, k_r, and K_e (the equilibrium constant for the isomerization) and minimizing χ^2 for the bromthymol blue data only. The constants obtained were $k_f = (6.5 \pm 0.2) \times 10^3$ s^{-1}, $k_r = (7.2 \pm 0.2) \times 10^4$ s^{-1}, and $pK_a = 5.67 \pm 0.05$. The solid lines in Figures 2–4 are theoretical curves calculated from eq 1 by using these values of the constants. The theoretical curves satisfactorily predict the concentration, indicator, and pH profiles for this indicator.

Having values for all of the constants, we should now be able to predict the behavior of the enzyme with other indicators. Since the value of α in eq 1 is dependent on the pK of the indicator, τ^{-1} values with different indicators will not necessarily be the same. Data are presented in Figures 2–4 for chlorophenol red and methyl red. The solid lines are again theoretical curves using the computer-fitted constants *as determined from bromthymol blue data alone*. The data for all three indicators are very well represented by a single, internally consistent set of constants. While the pH depen-

Table I: Rate and Equilibrium Constants for Enzyme Isomerizations

enzyme	k_f (s^{-1})	k_r (s^{-1})	K_e	pK_a	temp
ribonuclease A[a]	780	2468	0.32	6.1	25
	446	1823	0.24	6.1	10
carboxypeptidase A[b]	2710[e]	6610	0.41	5.68	25
	5760[f]	8340	0.69	5.85	
	159[g]	322	0.49	7.20	
aspartate aminotransferase[c]	1300	3420	0.38	6.25	22.5
hexokinase[d]	3000[h]	≥170000	≤0.018	≤5.5	15
	7000[h]	130000	0.054	5.8	
	6000[i]	160000	0.038	6.1	
deoxyribonuclease I	6500	72000	0.09	5.67	10

[a] Data of French & Hammes (1965). [b] Data of French et al. (1974). [c] Data of Giannini et al. (1975). [d] Data of Jentoft et al. (1977). [e] Fast isomerization (τ_1), Cox strain. [f] Fast isomerization (τ_1), Anson strain. [g] Slow isomerization (τ_2), Anson strain. [h] P_I isozyme, two different preparations. [i] P_{II} isozyme.

dences for chlorophenol red and bromphenol blue at the same enzyme concentration are not very different (the α values for these indicators being quite similar), the pH dependence for methyl red is strikingly different.

An isomerization process should be quite dependent on the structural integrity of the enzyme. Figure 5 shows the effect of urea on the amplitude of the relaxation process. The decrease in amplitude closely parallels the breakdown of the secondary and tertiary structure as monitored by the loss of enzymatic activity with increasing urea concentration.

Chemical modification of the ionizable group to which the isomerization is coupled with loss of the ionization should cause a complete loss of amplitude of the relaxation process. By using a number of different reagents known to react with specific residues on the enzyme, we may be able to determine the group to which the process is coupled. As a prelude to a more complete study, DNase I was alkylated under conditions such that a single histidine at the active site was modified. Figure 1 shows that the relaxation effect with alkylated enzyme (which had less than 5% of the activity of the native enzyme) was the same as that with native DNase I.

Discussion

Table I lists the five enzymes which to date have exhibited relaxational behavior attributed to an isomerization of the free enzyme. The mechanism for hexokinase, deoxyribonuclease, and carboxypeptidase A (τ_1) is the one originally proposed by French & Hammes (1965) for ribonuclease A: a slow isomerization of a protonated form of the enzyme coupled to a single fast proton transfer. For aspartate aminotransferase the enzyme isomerization is coupled to a rapid, two-proton cooperative transfer. The single example of an isomerization of a *deprotonated* form of an enzyme is τ_2 for carboxypeptidase A. The rate constants for the various isomerization processes vary considerably: k_f = 150–7500 s^{-1} and k_r = 300 to ≥ 170000 s^{-1}. The ratio k_f/k_r varies much less; with the exception of the value for hexokinase, it is on the order of 0.1–1. The effective pK_a values are, with one exception, all in the rather narrow range 5.5–6.3. The relaxation effects for the first four enzymes listed were found to be independent of enzyme and indicator concentrations. DNase is unique in that it is the only enzyme investigated for which the isomerization is clearly concentration dependent.

At this time, there is insufficient information to establish the role of this process in the overall catalytic cycle of the enzyme. We can, however, make several observations about the isomerization and its relation to other aspects of the enzyme's behavior. If the observed pK_a of 5.67 corresponds to the actual pK_a of a single ionizable group in the enzyme, the value is consistent with a histidine residue. From pH–activity profiles (Erkama & Sautarinen, 1949; Shank & Bynum, 1964) it has been established that the activity is dependent upon the deprotonated form of a group with a pK_a of about 6. Alkylation experiments have demonstrated an essential histidine necessary for activity (Price et al., 1969). However, the relaxation effect is still present in the enzyme which has been alkylated under conditions which modify only this essential histidine. This group is therefore not the one directly coupled to the isomerization. The fact that the isomerization is present in the alkylated enzyme, with no large shifts in τ^{-1} values, indicates that no gross structural changes have occurred upon reaction with iodoacetate. This supports the conclusion of Price et al. (1969) that this histidine is at the active site and not playing merely a structural role in maintaining the enzyme in the proper three-dimensional configuration for catalysis to take place.

We have demonstrated that at least two conformational forms of the enzyme exist in solution in the absence of substrate or cofactors. It is interesting to note that Poulos & Price (1972) have observed structural changes in the presence of divalent metal ions, especially Ca^{2+}. Changes involving the Ca^{2+} specific site and the active site were postulated. Further experiments involving the effects of metal ions and chemical modification of specific groups on the enzyme on the isomerization should help determine its importance in the overall mechanism.

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Sodium Binding Sites of Gramicidin A: Sodium-23 Nuclear Magnetic Resonance Study†

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ABSTRACT: In ethanol–water mixtures (90:10), the gramicidin dimer binds Na^+ cations at well-defined sites, with a binding constant $K = 4\ M^{-1}$. Partial desolvation of Na^+ occurs upon binding, as judged from the magnitude of the quadrupolar coupling constant (1.7 MHz) for bound sodium. The binding sites are identified with the outer sites flanking the channel entrances. The rate constants for binding and release are $k_+ \leq 2.2 \times 10^9\ M^{-1}\ s^{-1}$ and $k_- \leq 5.5 \times 10^8\ s^{-1}$, respectively.

Gramicidin, one of the very first antibiotics to be isolated (Dubos, 1939), greatly enhances the permeability of natural (Chappell & Crofts, 1965; Harold & Baarda, 1967; Harris & Pressman, 1967) and artificial (Mueller & Rudin, 1967) membranes toward monovalent cations. In fact, gramicidin is a mixture of six pentadecapeptides constituted by regularly alternating D and L residues, the main constituent being valine-gramicidin A (Gross & Witkop, 1965; Sarges & Witkop, 1964a,b, 1965a–d): formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-(L-Trp-D-Leu)$_3$-L-Trp-ethanolamide.

Ionic permeability conferred on membranes by gramicidin doping (Tregold, 1977) occurs through association of gramicidin into dimers which form channels (Bamberg & Lauger, 1973; Tosteson et al., 1968) responsible for the transport process. The two existing proposals for the channel-forming dimer, either head-to-head (Urry, 1971; Bamberg et al., 1977) or a double helical antiparallel β structure (Veatch & Blout, 1974), agree as to the length of the cylindrical channel, approximately 30 Å.

A recent model for the gramicidin A channel (Sandblom et al., 1977) includes a specific cationic binding site at each entrance to the channel. We adduce here direct evidence, from sodium-23 NMR measurements (Laszlo, 1978), for the existence of sodium-binding sites compatible with this proposal. Exchange of the sodium cations between these outer sites and the bulk solution is rapid compared with the transport process. We provide also a measurement of the rate constants for binding and release of Na^+ by these sites.

Experimental Procedure

Gramicidin, a gift from Société Rapidase (Seclin, France), has the composition: 70% gramicidin A, 10% gramicidin B, and 20% gramicidin C. We study its sodium-binding properties in ethanol–water binary mixtures (90:10) chosen both for solubility purposes and for the aqueous environment. Samples are prepared 24 h in advance from stock solutions containing either 100 or 50 mg of gramicidin/mL together with 10^{-2} M $NaClO_4$, diluted with 10^{-2} M $NaClO_4$ solutions. Ethanol (Baker Analyzed, reagent grade) has a water content of 0.05%. Sodium perchlorate (Merck, reagent grade) is dried under vacuum for 16 h immediately before use. Solvent stock mixtures are made by adding ethanol up to a final volume of 50.0 mL to 5.0 mL of deionized water. Samples are kept in the dark, at room temperature. After introduction in the NMR tubes, equilibration to the probe temperature (36 ± 1 °C) is ensured by waiting 15 min before the first spectrum is recorded. NMR spectra are obtained on Fourier transform instruments, at 23.81 MHz with a Bruker HFX-90 spectrometer field-locked on the deuterium signal of D_2O contained in a coaxial cell and at 62.86 MHz with a Cameca 250 spectrometer, without lock; 3500 to 95 000 transients are recorded, depending upon the conditions. All the NMR absorptions are Lorentzian and obey the criterion $w_{1/8} < 1.10 \times w_{1/2}\sqrt{7}$, where $w_{1/8}$ and $w_{1/2}$ are the line widths at $^1/_8$ and $^1/_2$ the total height (Delville et al., 1979).

Viscosities and densities are measured on these samples immediately after the NMR spectra have been recorded. Absolute viscosities are obtained from joint use of a Desreux-Bischoff viscosimeter (Desreux & Bischoff, 1950) and a pycnometer, both calibrated with water and thermostated at 36.0 ± 0.1 °C.

Results and Data Analysis

The sodium-23 line width in these experiments obeys the following set of equations applicable to transverse relaxation (Delville et al., 1979)

$$\frac{1}{T_2} = \frac{1 - p_B}{T_{2F}} + p_B\left[\frac{0.6}{T'_{2B} + \tau_B} + \frac{0.4}{T''_{2B} + \tau_B}\right] \quad (1)$$

$$\frac{1}{T'_{2B}} = \frac{\pi^2}{5}\chi^2\left[\tau_c + \frac{\tau_c}{1 + \omega^2\tau_c^2}\right] \quad (2)$$

$$\frac{1}{T''_{2B}} = \frac{\pi^2}{5}\chi^2\left[\frac{\tau_c}{1 + 4\omega^2\tau_c^2} + \frac{\tau_c}{1 + \omega^2\tau_c^2}\right] \quad (3)$$

where χ is the quadrupolar coupling constant (e^2qQ/h) for

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