Time-Resolved Analysis of Macromolecular Structures During Reactions by Stopped-Flow Electrooptics

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ABSTRACT A stopped-flow field-jump instrument and its use for the analysis of macromolecular structure changes during reactions is described. The operation of the new instrument is simple and reliable, owing to a new type of cell construction with electrodes directly integrated in a quartz cuvette: major advantages are the relatively low demand on sample quantities and a high time resolution. The stopped flow is characterized by a dead time of ~0.5 ms. Electric field pulses with field strengths up to 20 kV/cm and rise times in the nanosecond range are applied at adjustable times after stop of the flow. The time resolution of the optical detection is up to the nanosecond time range. The instrument may be used for the combination of stopped flow with temperature-jump and field-jump experiments. A particularly useful new application is the analysis of macromolecular reactions by electrooptical measurements, because electrooptical data provide information about structures. This is demonstrated for the intercalation of ethidium into double-helical DNA. The transients, measured at 313 nm, where the signal is exclusively due to ethidium bound to the DNA, demonstrate a relatively high negative dichroism at 0.5 ms after mixing. The absolute value of this negative dichroism increases in the millisecond time range and approaches the equilibrium value within about a second. The dichroism decay time constants demonstrate a clear increase of the effective DNA length due to ethidium binding, already 0.5 ms after mixing; a further increase to the equilibrium value is found in the millisecond time range. The analysis of these data demonstrate the existence of up to three relaxation processes, depending on the conditions of the experiments. The dichroism amplitudes, together with the decay time constants, indicate that all the reaction states found in the present investigation are complexes with insertion of ethidium residues between basepairs. Moreover, the data clearly show the degree of intercalation in the intermediate states, which is very useful information for the quantitative assignment of the mechanism.

INTRODUCTION

Stopped-flow electrooptics

One of the challenges in molecular biophysics is the identification of macromolecular structure changes occurring during reactions. Because reactions in biological systems are usually relatively fast, with time constants less than seconds, it is not trivial to identify these changes at the required time resolution. The standard techniques in reaction kinetics provide changes in spectroscopic parameters, which may be interpreted in terms of structure changes, but usually the signals do not provide direct information on the structure. Under these conditions it should be useful to develop techniques that can be used to get information on structures at a high time resolution. Among the techniques available for the characterization of macromolecular structures in solution, quantitative molecular electrooptics (Antosiewicz and Porschke, 1989; Porschke and Antosiewicz, 1990; Meyer-Almes and Porschke, 1997) proved to be particularly sensitive to details of structure changes, and furthermore is associated with a particularly high time resolution. Thus, it should be useful to combine the electrooptical technique with the stopped flow technique used in fast reaction kinetics. This combination requires a stopped-flow field-jump instrument that is closely related to that required for the combination of stopped flow and temperature jumps by Joule heating.

Soon after the first demonstration of the temperature jump technique as a very useful tool for the analysis of fast chemical reactions (Czerlinski and Eigen, 1959; Eigen and DeMaeyer, 1963), instruments have been constructed for combination of the stopped flow technique with temperature jumps (Erman and Hammes, 1966; Stewart and Lum, 1969; Faeder, 1970; Veil, 1975; Patel, 1976; Verkman et al., 1981). There are many important applications for these instruments, because the course of reactions is often determined by short lived intermediates; usually these intermediates cannot be isolated and, thus, their characterization is notoriously difficult. Relaxation techniques applied directly after mixing of the reagents should be very useful for the characterization of such intermediates. Despite the wide range of potential applications, the use of stopped-flow temperature-jump or of stopped-flow field-jump instruments remained very limited, which seems to be mainly due to technical difficulties. These difficulties were clearly illustrated to the author during attempts to get an instrument constructed by Veil (1975) into operation. Although a considerable amount of work was invested into improvements and reconstructions, it has not been possible to arrive at a state that would be satisfactory for standard applications. For example, it was quite difficult to get all the components

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of the instrument together without leak. The volume required for each shot was considerable and the time resolution was limited. During time-consuming attempts to reduce these difficulties, the concept for an alternative construction was developed. This concept proved to be productive without much difficulty as soon as the first measuring cell was assembled.

The new instrument may be used for stopped-flow temperature-jump and for stopped-flow field-jump experiments. A particularly attractive application is the analysis of reaction intermediates by electrooptical measurements. The case of ethidium intercalation into double helical DNA is used as an example.

Contradictory results on the reaction of ethidium with DNA

The intercalation reaction has been studied by many different groups (Magde et al., 1974; Bresloff and Crothers, 1975; Jovin and Striker, 1977; Wakelin and Waring, 1980; Mandal et al., 1980; Ryan and Crothers, 1984; Wilson et al., 1985; MacGregor et al., 1985, 1987; Monaco and Gardiner, 1993; Meyer-Almes and Porschke, 1993). Unfortunately, the conclusions are quite different. An extreme example is a recent paper by Monaco and Gardiner (1993), who did not get reproducible results for this reaction by temperature-jump measurements ("amplitude and relaxation time fluctuations") and declared the irreproducibility to be a "fundamental characteristics of the material itself." MacGregor et al. (1987) observed a single relaxation process and concluded that "intercalation is controlled by a diffusive process." Both the number of relaxation processes and the mode of reaction are controversial. Up to three relaxation processes have been reported in the literature. Most authors prefer the view that intercalation is a reaction-controlled process. Results supporting this view and a detailed discussion have been presented recently by Meyer-Almes and Porschke (1993).

It is quite remarkable that there is so much controversy about a reaction that appears to be rather simple. One of the reasons seems to be that the available techniques have not always been used with the appropriate care. However, it is also true that the available techniques do not always provide results which are sufficiently unambiguous. In particular, the available techniques do not provide information on the structures of reaction intermediates. As shown by the experiments described below, information about the changes of structure during the intercalation reaction is accessible by stopped-flow molecular electrooptics using the new instrument.

THE STOPPED-FLOW FIELD-JUMP INSTRUMENT

Construction of the cell

The main goals of the construction are as follows: 1) the volume required for each shot should be as small as possible, because large volumes are not accessible for most reagents of biological interest; 2) the time resolution should be as high as possible, in order to have a chance to characterize fast reactions; 3) operation of the instrument should be simple and reliable. The main technical problems associated with construction of a stopped-flow temperature/electric field-jump instrument result from the combination of different demands: fast flow requires application of a relatively high pressure; observation of the reaction by some spectroscopic technique requires appropriate windows, and finally electrodes are required for the induction of a temperature- or electric field-jump. The standard construction used in the past starts from the design of usual temperature-jump cells with a body machined from synthetic material like dynal. Quartz cones are inserted as windows for spectroscopic analysis and metal electrodes are screwed into the body. In the usual construction the metal electrodes include the channels for sample transport and the mixing chamber. The difficulties resulting from the combination of these elements are avoided according to the new concept by using a small quartz channel as the cell body. The flow of the mixed reactants is directed through this channel and the electrodes are deposited to the surface of the quartz as thin, conducting layers of gold or platinum.

The first construction of the cell was based on quartz plates with a vapor-deposited gold layer. It is known that gold does not stick to quartz and, thus, a "glue" in the form of a thin layer of chromium between the quartz and gold is commonly used for stabilization. Using this procedure, first a chromium layer of ~0.25 μm was vapor-deposited *around* quartz plates of $17 \times 6 \times 2$ mm^3 at a width of 6 mm in a distance of 6 mm from the one end. Then, a gold layer of ~0.5 μm was vaporized on the chromium layer. Two of these quartz plates were glued together with two quartz pieces of $17 \times 2 \times 2$ mm^3 to a small quartz cuvette with a channel leading straight through this cuvette (cf. Fig. 1 *a*). Because the metal layer was not more than 1 μm thick, the connection between the quartz pieces was not perturbed. Nevertheless, the metal layer was thick enough for a sufficient conductivity, such that a voltage applied to the outside of the cuvette was transmitted without any relevant Ohmic resistance to the inside.

This cell type has been used for a rather large number of experiments. After many shots, however, the thin electrode layers were damaged by electrolysis. Apparently the chromium "glue" was slowly dissolved through the gold layer, as must be expected according to the position of chromium in the electrochemical potential series. For this reason a corresponding cell has been constructed with electrodes from pure platinum. A thin leaf of platinum of 10 μm thickness was glued on the surface of $17 \times 6 \times 2$ mm^3 quartz plates, and then again two of these plates were combined with two $17 \times 2 \times 2$ mm^3 quartz pieces to a small cuvette with a straight through channel. The lifetime of this second cell type was clearly longer than that of the first one. However, the glue used for the assembly of the parts turned out to be of limited stability against repeated

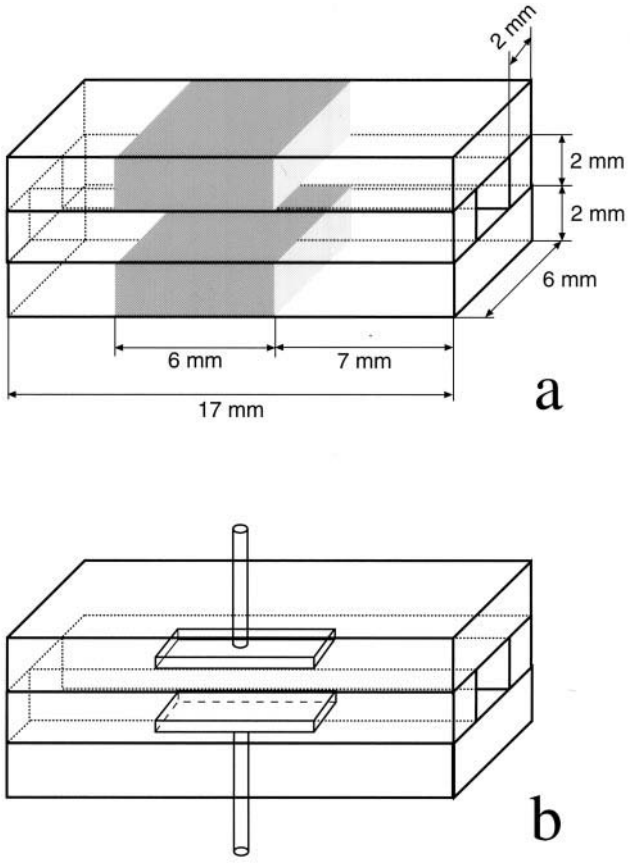

FIGURE 1 Cells for stopped-flow electric field-jump measurements. (*a*) Quartz cell with a thin metal layer around the upper and lower plates deposited from the gas phase; first a contact layer is made from chromium, which is then used as a basis for a gold layer. (*b*) Quartz cell with platinum electrodes integrated inside the upper and lower plates with electrical connections to the outside by platinum wires.

applications of high-pressure pulses, which are required for stopped-flow measurements with a high time resolution.

To avoid this problem, a third type of cell was constructed. In this case the quartz cuvette was "melted" together by Hellma GmbH and Co. (Müllheim, Germany) using a special technique developed by this company. The Pt-electrodes were inserted before the melting procedure into cavities prepared into the upper and lower plates forming the cuvette (cf. Fig. 1 *b*); electric connections to the outside are made by Pt-pins leading through small holes in the plates. After the quartz cuvette with the electrodes has been melted together, the small holes were closed by a glue. The cells have been assembled with special care in order to avoid strain in the quartz. This third type of cell is currently used without problems.

The cuvette channel is mounted in a small holder made from dynal (cf. Fig. 2) with windows for the optical detection and with two elastic contacts (gold-plated bronze), which provide the electric connection for the application of electric field pulses. This holder is mounted in a larger one (cf. Fig. 2), which is fixed into the stopped flow instrument.

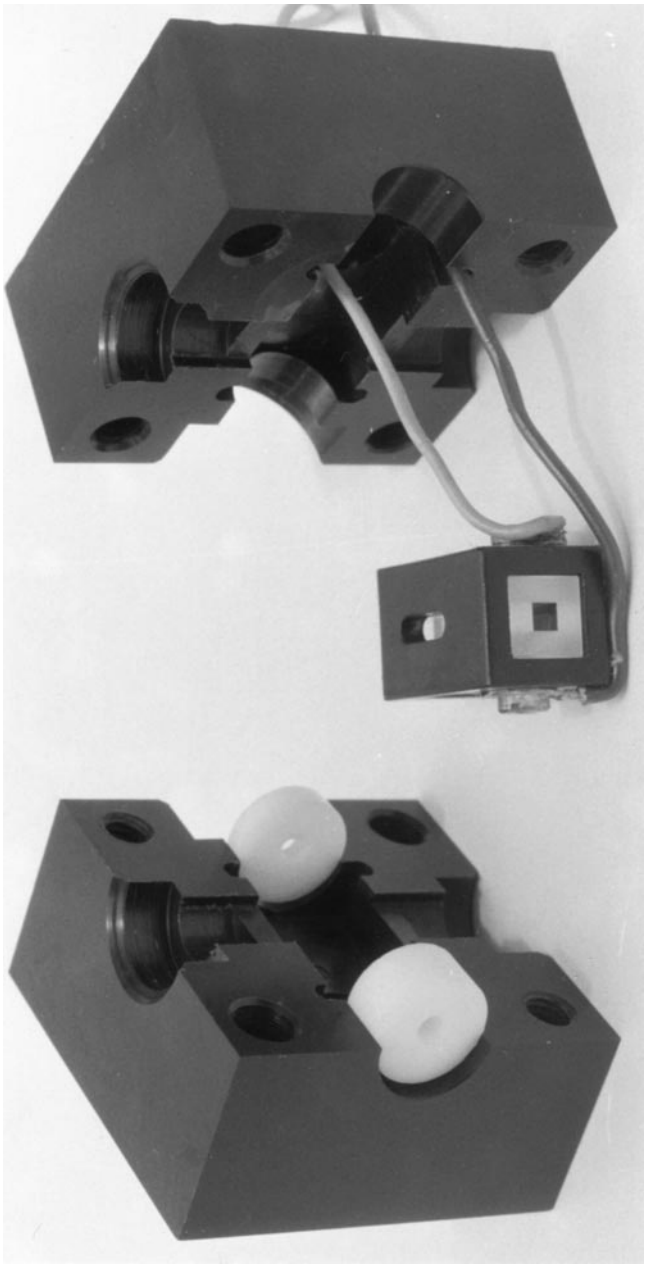

FIGURE 2 Mounting unit for the quartz cell. The inner mount is shown at the center, with windows for spectroscopic detection and with elastic contacts for electrical connections; the two parts of the outer mount (*top* and *bottom*) hold the inner mount in position for insertion into the stopped-flow instrument.

Stopped flow

The technical drawing of the stopped-flow instrument (cf. Fig. 3) shows the main parts and their assembly: the two syringes, the mixing chamber, the measuring cell, and the stop-syringe.

The optical detection is currently constructed from the following main components: a 200 W or a 500 W mercury-xenon lamp, a Bausch & Lomb high-intensity grating monochromator, and a photomultiplier with amplifier. For the measurements of the electric dichroism, a glan polarizer

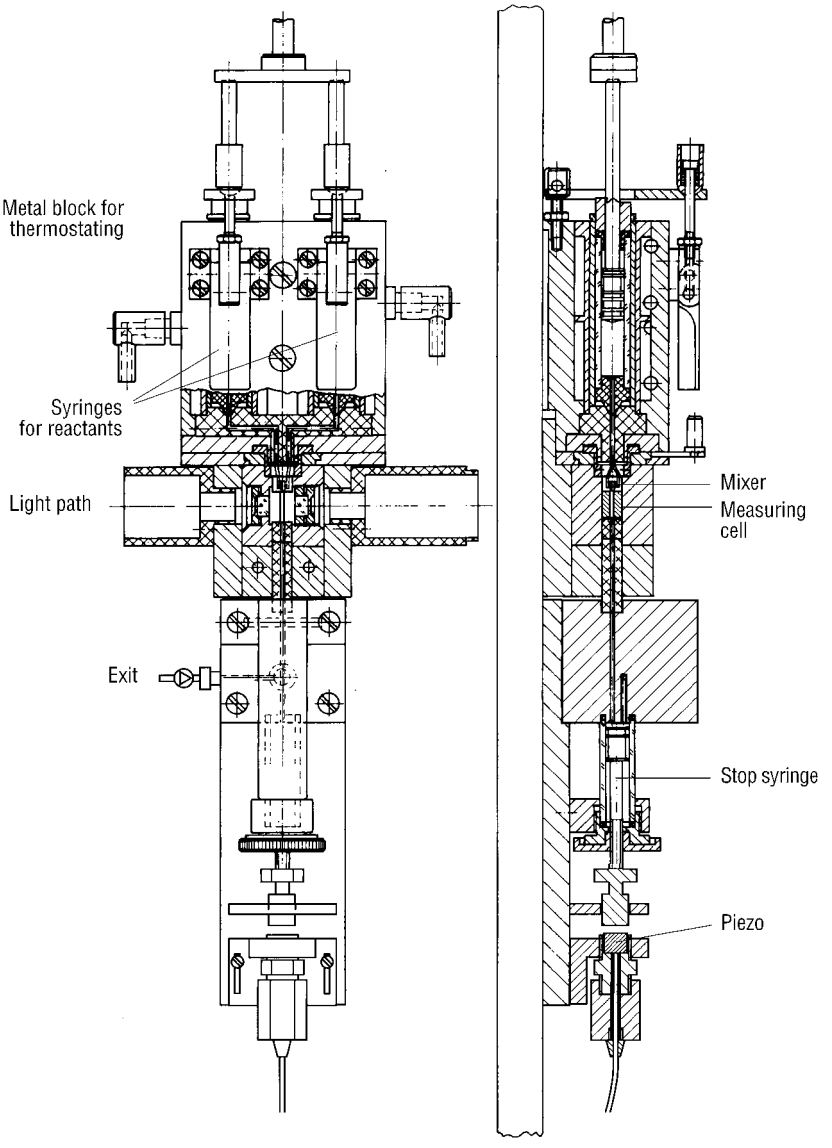

FIGURE 3 Construction of the stopped-flow instrument: top view (*left*) and side view (*right*).

made from calcite is inserted in the optical path before the cell. The measured signals are digitized by a Tektronix DSA602 and are transmitted to a PC.

Generation of electric field pulses

The piston of the stop syringe is stopped by impact on a piezo crystal in a small mounting unit. The electric signal delivered by the piezo crystal upon impact is transmitted to a time delay unit, which can be set to delay times starting from 0 in steps of 0.1 ms up to 999 s. The signal produced by the delay unit is then used to trigger a high-power pulse generator (model 606 from Cober Electronics Inc., Stamford, CN) with a trigger module P2T. Electric field pulses of high amplitude are generated by a series connection of two Cober 606; in this case a pulse generator 123A from E-H Research Laboratories Inc. (Oakland, CA) is used as a trigger module. The highest voltage, which has been applied in the present arrangement of the instrument, is 4 kV; this voltage results in an electric field strength of 20 kV/cm in the cell.

Performance of the instrument

The two most important criteria for the quality of a stopped-flow instrument are the homogeneity of mixing and the "dead time" of the flow. Mixing of a solution of zero absorbance with a solution of high absorbance did not lead to transients in the recorded signal; this result demonstrates that mixing into the measuring cell is completely homogeneous. The dead time was measured by the reaction of 2,6-dichlorophenolindophenol with ascorbic acid (Tonomura et al., 1978). By an excess of ascorbic acid the reaction was forced to be of pseudo-first-order; the reduction reaction of 2,6-dichlorophenolindophenol was recorded at 580 nm. Measurements with ascorbic acid concentrations in the range from 10 to 50 mM showed that the dead time of the instrument under the standard conditions of operation is 0.5 ms.

The performance of the combined stopped-flow field jump instrument (cf. Fig. 4) is demonstrated by the examples given in the following section. The accuracy of the time constants for the 95 bp DNA fragment is estimated to be $\pm 1.5\%$; the accuracy of the amplitudes is $\sim \pm 0.6$ mV.

MATERIALS

Ethidium bromide was obtained from Merck (Darmstadt, Germany) and was used without further purification. The concentrations of stock solutions were evaluated from measurements of the absorbance at 480 nm using an extinction coefficient of 5700 M^{-1} cm^{-1}. The fragment with 95 basepairs was prepared from a plasmid DNA (pRW574, Hillen et al., 1981) by digestion with *Eco*RI restriction endonuclease. The fragment was isolated by Sepharose 4B column chromatography. In its final state the fragment proved to be homogeneous according to polyacrylamide gel electrophoresis. The DNA fragment was dialyzed extensively against the buffers used for the experiments. The DNA concentrations are given in units of mole basepairs per dm^3 (determined by measurements of the absorbance at 260 nm using an extinction coefficient of 13×10^3 M_{bp}^{-1} cm^{-1}). Buffer A contained 1 mM NaCl, 1 mM sodium cacodylate pH 7.0, and 100 μM $MgCl_2$; buffer B contained 1 mM NaCl, 1 mM sodium cacodylate pH 7.0, and 200 μM EDTA.

RESULTS

Stopped-flow electrooptical experiments on the reaction of ethidium with DNA

The reaction of ethidium with double helical DNA may be recorded by electrooptical measurements at wavelengths either in the spectral range, where the DNA contributes to the absorbance, or outside the absorbance range of DNA using the absorbance of ethidium. An example of a transient recorded at 248.2 nm (Fig. 5), where DNA contributes much more to the absorbance than ethidium, demonstrates that the signal-to-noise ratio is quite satisfactory. Dichroism signals measured at 313.0 nm can only be due to ethidium bound to the DNA, because the electric dichroism of free ethidium is zero and that of DNA at this wavelength is also virtually zero. Thus, it is of special interest to characterize the dichroism amplitudes at this wavelength at various times of the reaction. As shown for the example of a 95-bp restriction fragment in Fig. 6, the dichroism amplitude increases in the millisecond time range. An exponential fit of these amplitudes demonstrates a relaxation process with a time constant of 3.6 ms. The rather large dichroism, which is observed before the onset of this relaxation process, clearly demonstrates the presence of another process with a lower time constant and the existence of an intermediate species I characterized by a negative dichroism.

The observation of two relaxation processes for an intercalation reaction suggests an interpretation in terms of a mechanism with a fast binding process to the "outside" followed by a slow insertion reaction. The electric dichroism can be used to check whether the intermediate corresponds to an "outside" complex (cf. following section). Further information about the structure of the intermediate is obtained from the dichroism decay time constants shown in Fig. 7. The progress of the intercalation reaction in the millisecond time range is clearly reflected by an increase of the dichroism decay time constants; an exponential time constant fitted to these data ($\tau = 4.0$ μs) closely corresponds to that obtained from the dichroism amplitudes. The exponential fit also reveals the dichroism decay time constant at the start of the relaxation process, 1.179 μs, which is clearly larger than that of the free DNA fragment. Thus, the decay time constants again indicate the existence of a fast relaxation process and the formation of an intermediate. As shown in the Discussion, the magnitude of the decay time constant indicates that the intermediate already repre-

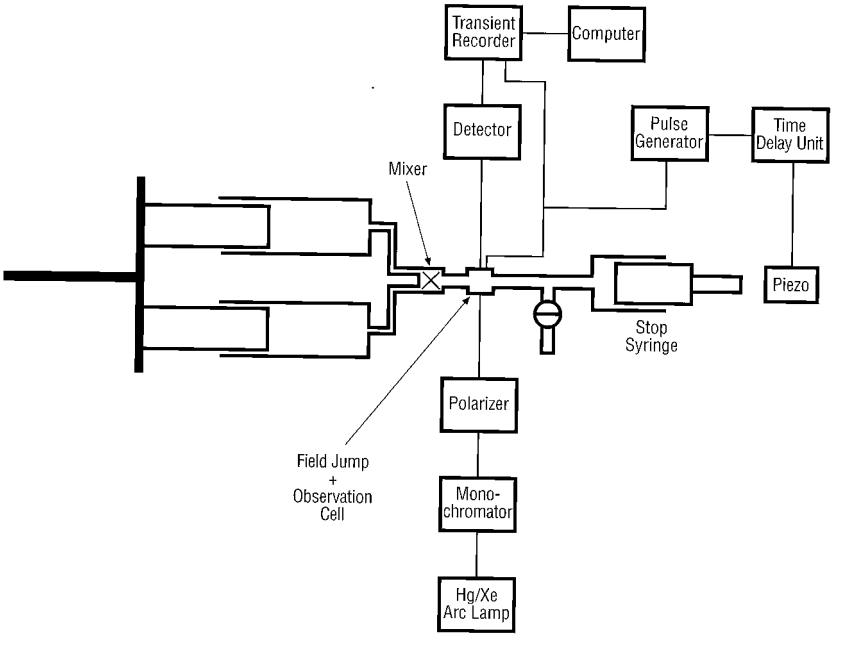

FIGURE 4 Block diagram of the stopped-flow field-jump instrument.

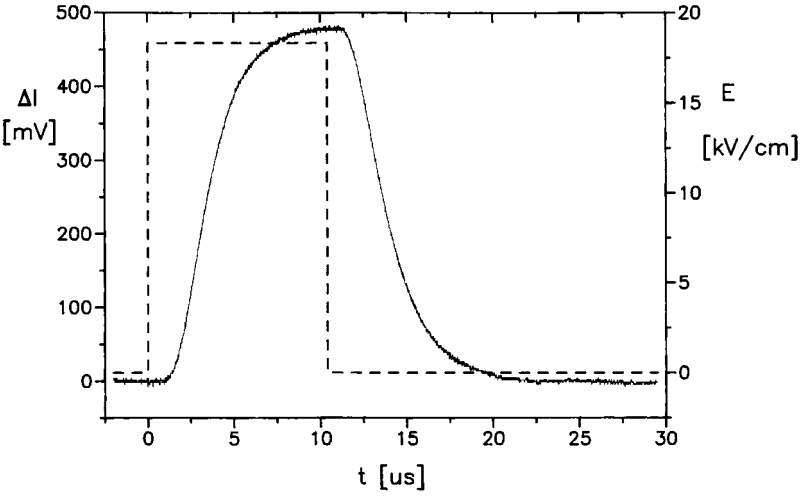

FIGURE 5 Electric dichroism transient measured at 248.2 nm for a mixture of 50 μM_{bp} 95-bp fragment and 10 μM ethidium in buffer A at 10°C at a time delay of the pulse 2 ms after stop of the flow. The delays of the optical responses after pulse application/termination and the deviations from the single exponential form are due to convolution with the detector response function; the detector has been adjusted to a relatively slow response in order to increase the signal to noise ratio. Because the detector response function can be measured at a high accuracy, deconvolution is without problems (Porschke and Jung, 1985). The temperature jump induced by the field pulse is calculated to be 0.13°C.

sents a species with insertion of aromatic residues into the double helix.

The experimental data given in Figs. 6 and 7 were obtained at a DNA concentration in a clear excess over that of ethidium, in order to simplify the reaction 1) by pseudo-first-order conditions and 2) by avoiding excluded site effects (McGhee and von Hippel, 1974; Epstein, 1979). As a control, the reaction has also been studied at a still larger excess, using 50 μM_{bp} DNA and 5 μM ethidium in the mixture. In this case the electrooptical transients were recorded at 280.4 nm, where the DNA shows an electrooptical signal, which is increased upon reaction with ethidium. The results are very similar to those described above: a slow process was found with a time constant of 4.0 ms; the dichroism increase associated with this process was 63% of the total increase; 37% of the dichroism increase is associated with the fast process. This result confirms that the observation of two relaxation processes is not due to deviations from pseudo-first-order conditions and also not due to excluded site effects.

The new technique may be used to study various problems associated with the intercalation reaction. One of them is the dependence on the solvent conditions. The buffer A used for the experiments described above contains Mg^{2+} ions and thus the electrostatic contribution to the binding affinity of ethidium is close to that found in 0.1 M monovalent salt concentrations (publication of data in preparation). When the Mg^{2+} ions are replaced by EDTA (buffer B), the electrostatic contribution to the binding affinity is clearly higher. An analysis of the reaction mechanism under these conditions reveals a modified reaction pattern. The dichroism amplitudes measured at 313.0 nm (Fig. 8) show an increased contribution by the first "fast" process, which is followed by two processes in the millisecond time range

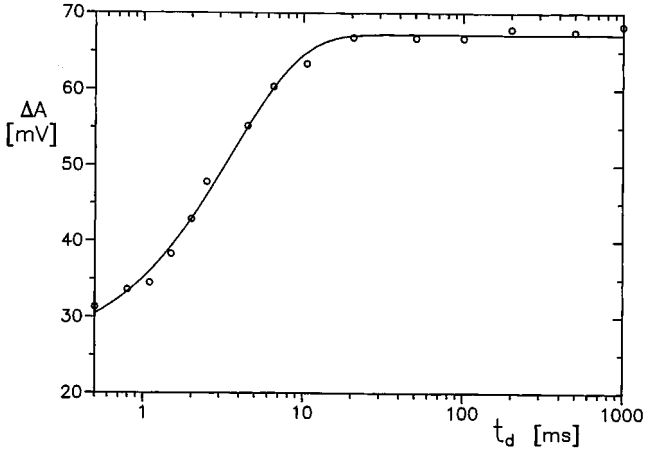

FIGURE 6 Dichroism amplitudes ΔA measured at 313.0 nm for 50 μM_{bp} 95-bp fragment and 10 μM ethidium in buffer A as a function of the time delay after mixing t_d (time delay after stop of the flow + dead time of the stopped flow, 10°C, 12.5 kV/cm). Each of the experimental points represents the results obtained from an average of two stopped-flow shots, using single field pulses per shot with pulse lengths of 10 μs. The line represents a least-squares fit of the data by a single exponential of 3.6 ms.

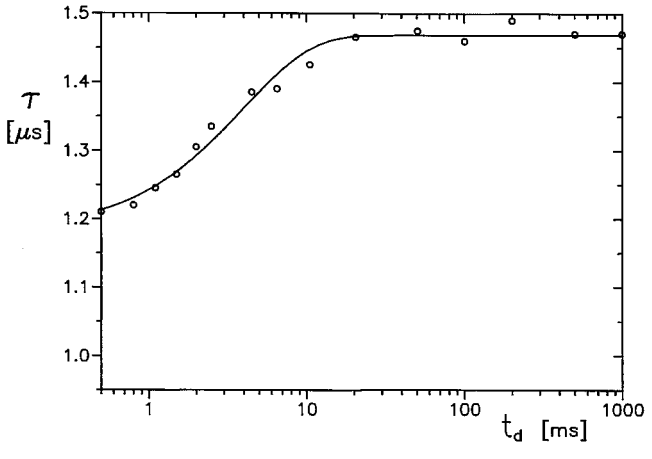

FIGURE 7 Dichroism decay times τ measured at 248.2 nm for 50 μM_{bp} 95-bp fragment and 10 μM ethidium in buffer A as a function of the time delay after mixing t_d (10°C, 12.5 kV/cm). Each of the experimental points represents the results obtained from an average of two stopped-flow shots, using single field pulses per shot with pulse lengths of 10 μs. The line represents a least-squares fit of the data by a single exponential of 4.0 ms [$\tau(t_d = 0) = 1.179$ μs and $\tau(t_d = \infty) = 1.469$ μs].

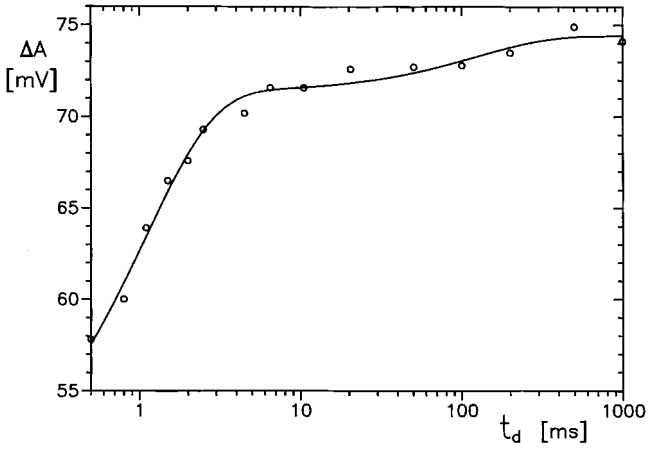

FIGURE 8 Dichroism amplitudes ΔA measured at 313.0 nm for 50 μM_{bp} 95-bp fragment and 10 μM ethidium in buffer B as a function of the time delay after mixing t_d (10°C, 12.5 kV/cm). Each of the experimental points represents the result from a stopped-flow shot, using a single pulse per shot with pulse lengths of 14 μs. The line represents a least-squares fit of the data by exponentials of 1.1 ms and 120 ms.

with exponential time constants of 1.1 and 120 ms. The dichroism decay time constants measured under the same conditions indicate that most of the length increase resulting from intercalation is associated with the first relaxation process (~80%).

The reaction of ethidium with DNA has been studied previously by other techniques like measurements of the fluorescence increase upon intercalation. Thus, it is of interest to check the relaxation time constant(s) observed by fluorescence measurements under the experimental conditions used for the measurements of the electric dichroism. Control experiments using stopped-flow with fluorescence detection confirm the existence of the relaxation processes, which have been identified above. The main advantage of the stopped-flow experiments with electrooptical detection is the information provided on the structure of the intermediates.

Interpretation of the electrooptical data

The interpretation of electrooptical data is relatively simple for molecules with a simple shape. In this respect DNA molecules are simple, if their length remains below the "persistence length." The DNA fragment with 95 bp has been selected for the present experiments, because its length is below the persistence length of ~500 Å ≡ ~150 bp (in buffer A). The 95-bp DNA may be considered as a rodlike molecule, which is reflected by the fact that the dichroism decay of this fragment can be described by a single exponential. The rotational diffusion time constant of such molecules increases approximately with the cube of their length (Tirado et al., 1984) and, thus, intercalation can be detected at a high sensitivity due to the increase of the length. Changes of the diameter of the rod are almost without effect on the time constant of rotational diffusion.

Two modifications of the simple interpretation described above should be considered for a sufficiently accurate description of the experimental data:

1. The 95-bp fragment bears four single-stranded residues at each end, resulting from the preparation by *Eco*RI restriction nuclease. Independent experiments showed that these residues contribute to the effective hydrodynamic length by an equivalent of 5 bp (Porschke et al., 1988). Thus, the effective hydrodynamic chain length of the fragment is 100 bp.
2. Although the DNA fragment is relatively short, some flexibility is already detectable in its time constant of rotational diffusion. In the absence of any flexibility, the rotational time constant of a 100-bp DNA fragment is expected to be 1.16 μs (rigid rod of length 100 × 3.4 Å and radius 13 Å at 10°C; cf. Porschke, 1991). The rotational time constant measured at 10°C in buffer A is 0.99 μs. According to the wormlike coil model this corresponds to a persistence length of 610 Å, which is in the expected range under the given experimental conditions (Porschke, 1991). All the calculations according to the wormlike coil model were based on the combination of the rotational diffusion coefficients for cylinders given by Tirado et al. (1984) with correction factors derived from Monte Carlo simulations by Hagerman and Zimm (1981).

Independent experiments demonstrated that the persistence length of ethidium-DNA complexes is close to that of free DNA (D. Porschke, manuscript in preparation). Thus, the persistence length of 610 Å will be used below for further model calculations, which are required for the interpretation of the kinetic data.

The standard mechanism used for the intercalation reaction assumes that the aromatic residues are first bound to the "outside" of the double helix in a relatively fast reaction step according to

$$\text{DNA} + \text{ethidium} \rightleftharpoons \text{OC} \qquad (1)$$

In a relatively slow second step the "outside" complex (OC) is converted into an inside complex (IC) with the aromatic residues inserted between basepairs

$$\text{OC} \rightleftharpoons \text{IC} \qquad (2)$$

The experimental data obtained by stopped flow electrooptics can now be used to check whether the intermediate I observed in the experiments (cf. previous section) corresponds to the OC, postulated to be formed in the first reaction step (1). "Outside" complexes are expected to be characterized by maximal contacts of the aromatic residues to the outside of the DNA, which should lead to a more or less parallel orientation of the aromatic planes to the helix axis. For this kind of orientation the electric dichroism may have any value, but usually is found to be positive (Colson et al., 1996). The observed dichroism is clearly negative, suggesting an orientation of the aromatic residues parallel to

the base planes. Thus, it is not likely that the intermediate is an outside complex.

The structure of the intermediate is more clearly demonstrated by its dichroism decay time constant. According to the data given in Fig. 7 the rotational time constant of the intermediate is 1.179 μs, which is equivalent to a hydrodynamic length of 109 bp, indicating that nine ethidium molecules are intercalated on average. The final state of the reaction is characterized by a rotational time constant of 1.469 μs, which corresponds to a hydrodynamic length of 120 bp and, thus, to intercalation of 20 ethidium molecules. At the given input ethidium/DNA ratio, all ethidium molecules are intercalated in the final state of the reaction. According to the independently determined overall binding constant (D. Porschke, manuscript in preparation), at equilibrium virtually all ethidium molecules are bound to the DNA under the conditions of the experiments.

As a next step we may check whether the change of the dichroism amplitude is approximately parallel to the number of intercalated ethidium molecules derived from the time constants. According to the data in Fig. 6, the dichroism amplitude found for the intermediate is 37% of that for the final state, which is equivalent to the ratio of intercalated ethidium molecules (9/20 $\equiv$ 45%) within experimental accuracy.

In the present investigation the dichroism transients have always been recorded at a single electric field strength and thus it is not possible to evaluate the limiting value of the dichroism corresponding to complete orientation. Nevertheless, it is useful to compare the dichroism values of ethidium in its DNA-complexes at the given field strength with the value obtained for the DNA alone at the same field strength. These dichroism values $(\Delta\varepsilon_{\parallel} - \Delta\varepsilon_{\perp})/\varepsilon$ are: for state *I* -0.10, for the final complex -0.27 (both values at 313.0 nm using the total absorbance of ethidium); for the free DNA -0.19 (at 248.2 nm). In the comparison of these values one has to consider that the ethidium-DNA complexes are clearly longer than the DNA, which is expected to increase the orientation function. This appears to be the main reason for the rather large dichroism of the final complex compared to that of the free DNA.

According to all these data, the intermediate *I* represents a state of the reaction where ~40% of the available ethidium molecules are already intercalated into the double helix. Most of the remaining 60% of the available ethidium molecules are likely to be free in solution during this state of the reaction. The fraction of ethidium molecules bound to the DNA in the form of OC appears to be negligible. It is likely that OC are mainly stabilized by electrostatic interactions, whereas other contributions are expected to remain relatively small. Under these conditions the binding constant for OC is expected to be small ($>10^3 M^{-1}$). This is consistent with the present experimental data, which do not indicate any significant population of an intermediate with a positive electric dichroism.

The experimental conditions in buffer A are similar to those used in previous investigations of the mechanism of ethidium intercalation, at least with respect to the relative contribution of electrostatic interactions. According to the results of some of the previous investigations (Bresloff and Crothers, 1975; Wakelin and Waring, 1980; Meyer-Almes and Porschke, 1993), there are two different complexes formed by ethidium with DNA double helices. The present results clearly confirm that there are two complexes formed at different rates, and furthermore demonstrate that in both complexes ethidium is intercalated between basepairs. Thus the intercalation reaction is clearly more complex than has been recognized by some of the techniques applied previously, like pressure jump (MacGregor et al., 1987).

An even more complex reaction scheme is demonstrated by the data obtained in buffer B, where an additional process is clearly indicated (cf. Fig. 8). These results raise various questions, which can only be answered by further experiments. A detailed discussion of the problems associated with the intercalation reaction of ethidium into DNA together with new results will be presented elsewhere (D. Porschke, manuscript in preparation).

DISCUSSION

The new instrument may be used for different types of experiments, including stopped-flow temperature jump or stopped-flow field jump experiments. Electrooptical applications are of special interest, because electrooptical signals provide information about structures in solution at a high sensitivity. The number of reactions that may be analyzed by this new technique is very large. A particularly attractive class of reactions is the binding of various types of ligands to nucleic acids, because nucleic acids show particularly high electrooptical signals. One of the advantages of the technique is the fact that reactions can be indicated even in the absence of standard spectroscopic signals like fluorescence or absorbance changes. For example, the binding of proteins to nucleic acids in general and the winding of double helical DNA around proteins in particular are directly accessible to analysis, without introduction of spectroscopic labels. Furthermore, applications are clearly not restricted to nucleic acids: there are many other systems of general interest, including membrane proteins like bacteriorhodopsin or Na^+/K^+-ATPase, where new types of experiments are now possible. Most of the reactions of biological interest are relatively fast and, thus, their analysis requires the extension of the time resolution provided by stopped-flow techniques. Few reactions are sufficiently slow such that measurements of the kinetics may be initiated by simple mixing of the reagents in standard cuvettes (cf. Amiri and Hagerman, 1996).

In the present investigation the electrooptical approach has been demonstrated by the example of the well-known intercalation reaction of aromatic compounds into the double helix. This reaction has been analyzed previously by various spectroscopic techniques; all these techniques provide a signal demonstrating the progress of the reaction, but

none of these signals involves information about the structure. The electrooptical approach has not been used here to determine relaxation time constants over a broad range of concentrations. This type of studies may be conducted by the standard techniques developed previously. The most important contribution of the new technique is the characterization of individual relaxation processes in terms of structures of intermediates. In the present case it has been possible to demonstrate that an intermediate found during the reaction in buffer A corresponds to an intercalated structure. Moreover, it has been shown how many ethidium molecules are intercalated in this intermediate state. This information is very useful for the quantitative interpretation of data obtained by experiments with fluorescence detection (cf. Bresloff and Crothers, 1975; Wakelin and Waring, 1980; Meyer-Almes and Porschke, 1993). The amplitudes obtained from fluorescence experiments can only be interpreted by introduction of optical parameters, which are usually subject to a considerable uncertainty.

The outside complex, which is expected as an intermediate, has not been identified yet. It is very likely that the OC has a relatively short lifetime and, furthermore, that it is formed at a rather low concentration under the usual experimental conditions. The number of reaction steps detected in the present investigation and the assignment of these steps is consistent with the general mechanism derived previously by Meyer-Almes and Porschke (1993) from temperature-jump and stopped- flow measurements with fluorescence detection. This mechanism has been proposed first by Bresloff and Crothers (1975), but has been questioned in several subsequent publications (MacGregor et al., 1985, 1987; Monaco and Gardiner, 1993). The problems in the assignment of the mechanism appear to be due to various sources. A more detailed discussion of these problems associated with the intercalation mechanism together with new experimental data will be presented elsewhere (D. Porschke, manuscript in preparation).

In the present investigation the utility of stopped-flow electrooptics has been demonstrated by measurements in a narrow range of reactant concentrations, which was determined by the optical path-length of the cell. Construction of cells with higher optical path-lengths should not be a serious problem and will extend the range of concentrations, where large electrooptical signals can be obtained. A particularly useful extension of the potential will be provided by fluorescence-detected stopped-flow electrooptics, which is also under construction.

The case of ethidium intercalation into DNA has been selected as a first application of the new technique because it is a well-known reaction that has been investigated previously by many different groups and different techniques. The selection of this reaction as an example does not imply that the new technique can only be applied to cases where "unlimited" amounts of reactants are available. One of the advantages of the instrument is the relatively low demand on sample quantities: a single shot is usually done with $\sim$150 μl mixed solution; it is possible to use smaller volumes. This feature has already been useful during the present investigation, because the use of restriction fragments would not be possible otherwise; obviously the data can be interpreted into much more detail when collected for well-defined systems. Another reaction that has already been studied successfully by the new technique is the binding of a gene-activating protein, the cyclic AMP receptor, to an operator DNA restriction fragment. Thus, it is possible to study reactions with reactants that are not so easily accessible in large quantities.

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