Biophysical Chemistry 16 (1982) 193–198
Elsevier Biomedical Press

DIFFERENCES IN THE ELECTRIC BIREFRINGENCE OF SPECTRIN DIMERS AND TETRAMERS AS SHOWN BY THE FAST REVERSING ELECTRIC PULSE METHOD

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Received 8th January 1982
Revised manuscript received 8th June 1982
Accepted 16th June 1982

Key words: Spectrin; Electric birefringence Dipole electric moment; Reversing electric pulse method

The electric birefringence of purified spectrin has been examined in medium of low ionic strength at 20°C and for electric fields smaller than 4×10^4 V m^{-1}, using the reversing electric pulse method. This technique allows study of the permanent and induced dipole electric moment of macromolecules more easily than in measurements using only rectangular pulses. We show that spectrin heterodimers and heterotetramers have different electro-optical properties. The relaxation time of the tetramer (7 μs) is significantly longer than that of the dimer (4.5 μs). Tetramers and dimers have also different polarizability parameters.

1. Introduction

Mikkelsen and Elgsaeter [1,2] have studied static and transient electric birefringence of spectrin heterodimers and heterotetramers by applying a rectangular electric pulse across the solution. We describe here results obtained with a different method, i.e., by use of reversing electric pulses. The use of rapid reversing pulses to study electric birefringence of macromolecule solutions has been proposed earlier [3,4]. This method is a powerful tool for obtaining the relative contribution of the induced and permanent dipole moment of macromolecules. On the other hand, very low strength electric fields are applied across the solution ($E < 4\times10^4$ V m^{-1}); so, contrary to Mikkelsen and Elgsaeter [2] who used rectangular pulses of high strength ($E > 3\times10^5$ V m^{-1}), we have found some differences between spectrin heterodimers and heterotetramers.

2. Materials and methods

2.1. Preparation of spectrin heterodimers and heterotetramers

Freshly drawn human blood was washed six times in isotonic NaCl solution. Unsealed ghosts were prepared from the packed cells by the method of Dodge et al. [5] with the slight modification that hemolysis was conducted in the presence of 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. Spectrin dimers were extracted from the ghosts in 0.5 mM phosphate buffer, pH 8, at 35°C for 30 min before chilling rapidly to 0°C. To extract the tetramers a suspension of ghosts was dialyzed extensively in the cold room against the same buffer. All the subsequent operations were made at 2°C in order to prevent interconversion of the two forms of spectrin. After centrifugation for 1 h at 50000 *g*, the supernatants were concentrated under vacuum and filtered on Sepharose 4B columns equilibrated with 10 mM Tris, 100 mM NaCl, 5 mM mercaptoethanol, 1 mM NaN_3. Spec-

0301-4622/82/0000–0000/$02.75

trin tetramers and dimers were eluted from the columns. In the former case, special care was taken to collect only the second part of the elution peak in order to avoid contamination of the tetramer with aggregated forms of spectrin which are usually eluted with traces of actin in the void volume of the columns. The fractions were concentrated under vacuum up to 1 mg/ml, dialyzed against 5 mM phosphate buffer, pH 8, and stored in the cold room. The purity and integrity of the preparations were controlled each time by sodium dodecyl sulfate polyacrylamide gel electrophoresis. When the gels were overloaded with spectrin tetramers they did not show traces of contamination. Controls were also made by velocity sedimentation at 20°C on a Spinco analytical ultracentrifuge to assess the dimeric or tetrameric state of spectrin. In the latter case, the absence of spectrin aggregates was further demonstrated by the absence of rapidly sedimenting material within the time necessary for the rotor to reach the speed of the run. Spectrin concentration was calculated per dimer on the basis of M_r 460000 and $A^{\%}_{280} = 10.6$ [6]. Electric birefringence measurements were performed at 20°C in 5 mM phosphate, pH 7.4. Under these conditions, during the time of the experiments which only lasted a few minutes after the samples had been equilibrated at 20°C, spectrin dimers and tetramers were stable as their interconversion is achieved on a larger time scale at this temperature. Ungewickel and Gratzer [7] have indeed shown, although the dissociation constant of the tetramer-dimer equilibrium is considerably increased by decreasing the ionic strength, that this reaction is associated with a very high activation energy even at low ionic strength. We have confirmed this result by analytical ultracentrifugation controls performed at 20°C. We can thus be confident that during the time course of the birefringence experiments spectrin tetramers are stable entities.

2.2. Electric birefringence measurements

The principle of electric birefringence is to orientate macromolecules by mean of a rectangular electric field pulse [8]. The resulting birefringence Δn_{eq} is proportional to the square of the electric field, at low field strength. The specific Kerr constant is defined by:

$$B = \Delta n_{eq} / C\lambda E^2$$

where C is the solute concentration and λ wavelength of the incident light used to determine the birefringence.

The rise of birefringence $\Delta_B(t)$ depends on the value of E and the molecular electric properties. The birefringence decay $\Delta_D(t)$ depends only on the hydrodynamic properties of the solute. Formulae used in our work are well known [8]; they are valid only for cylindrical models. If the solution is polydisperse:

$$\Delta_B(t) = 1 + \sum_i a_i \left[\frac{r_i - 2}{2(r_i + 1)} e^{-t/\tau_i} - \frac{3r_i}{2(r_i + 1)} e^{-t/3\tau_i} \right] \quad (1)$$

$$\Delta_D(t) = \sum_i a_i e^{-t/\tau_i} \quad (2)$$

where $\Delta_B(t)$ and $\Delta_D(t)$ are normalized birefringence (with respect to Δn_{eq}) and the index i is related to the molecular entity of relaxation time τ_i.

In these equations:

$$a_i = \frac{\Delta n_i}{\sum_i \Delta n_i}$$

and r_i characterizes the electric properties of the macromolecules:

$$r_i = \mu_i^2 / \Delta\alpha_i kT \quad (3)$$

μ_i being the permanent dipole moment (lying along the main axis for a cylinder) and $\Delta\alpha_i$ the anisotropy of electrical polarizability [8].

The relaxation time τ_i is related to the rotational diffusion constant D_i by

$$\tau_i = 1/6D_i \quad (4)$$

D_i depends on the size of the molecules according to the well known Broersma equation [8]

$$D_i = \frac{3kT}{\pi\eta L_i^3} \left[\ln\left(\frac{L}{b}\right)_i - 1.57 + 7\left(\frac{1}{\ln\left(\frac{L}{b}\right)_i} - 0.28 \right)^2 \right] \quad (5)$$

which is valid for a cylindrical molecule of length

L_i and radius b_i, η being the solvent viscosity and kT the Boltzmann term.

In a rapid reversing field, it is possible to determine more easily the same parameters by the following equations

$$\Delta_R(t)=1+\sum_i a_i\left(\frac{3r_i}{r_i+1}\right)(e^{-t/\tau_i}-e^{-t/3\tau_i}) \quad (6)$$

If the solution contains a single species $\Delta_R(t)$ has an extremum Δn_m at time t_m:

$$t_m=\ln 3/4D \quad (7)$$

which allows easy calculation of the ratio r from the relationship:

$$r=(1-\Delta n_m)/(0.1547+\Delta n_m). \quad (8)$$

Electric birefringence measurements were performed on an apparatus built in our laboratory by J.C. Bernengo [9].

The light source was an He-Ne laser ($\lambda = 632.8$ nm) and light beam variations were converted to electric signals by a low-noise state photodetector followed by a specially designed amplifier. The signal intensity is linearly proportional to the birefringence. Rectangular and reverse electric pulses having amplitudes up to 200 V were applied across a 0.5 cm interelectrode cell, with a duration of about 400 μs and transition times of less than 1 μs. A single pulse was used and the birefringence signals were sampled on a transient recorder. The curves were analyzed through a computer program based on a two-step adjustment fitting [10]. In the present case, only one relaxation time is necessary to describe the relaxation process.

3. Results

The field dependence of the electric birefringence of spectrin is depicted in fig. 1. The specific Kerr constants calculated from the slopes of the lines are 4.1×10^{-5} and 4.9×10^{-5} m V^{-2} M^{-1}, respectively, for spectrin heterodimers and tetramers. The birefringence is positive and specific values are concentration independent between 0.5 and 4 mg/ml.

Fig. 2 shows the variation of birefringence with time when a reversing electric pulse is applied to a

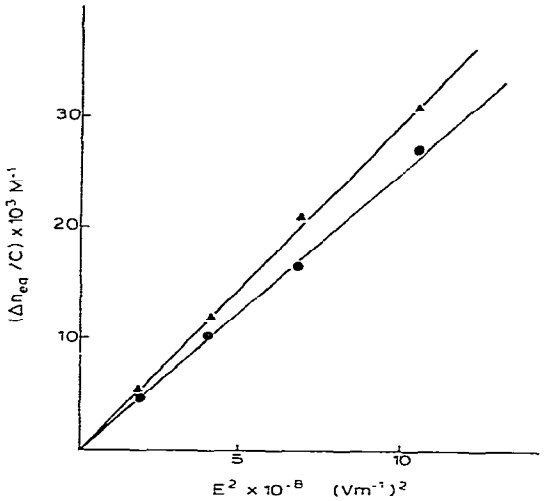

Fig. 1. Specific birefringence vs. E^2 for spectrin dimer (●) and tetramer (▲). Concentrations are 8.2×10^{-7} M (per dimer). Buffer is 5 mM phosphate, pH 7.4, at 20°C. The slopes are 2.6×10^{-11} m^2 V^{-2} M^{-1} for dimer and 3.1×10^{-11} m^2 V^{-2} M^{-1} for tetramer.

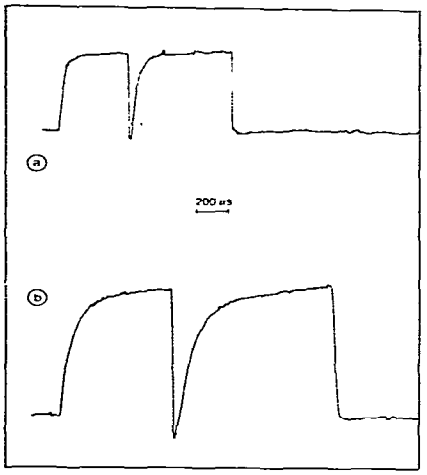

Fig. 2. Variation of birefringence vs. time, when a reversing pulse is applied ($E=2.6\times10^4$ V m^{-1}). The ordinates are arbitrary units. The curves show a significant difference in the minimum values of the birefringence obtained when the pulse is reversed. They give r values of opposite signs for spectrin dimers (a) and tetramers (b). To obtain the value of Δn_{eq}, the electric pulse length applied to the tetramer solution must be longer than that used for the dimer.

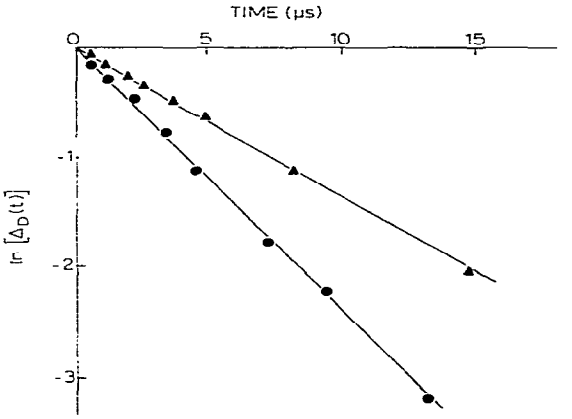

Fig. 3. Birefringence decay of spectrin dimer (●) and tetramer (▲). The conditions are those of fig. 1 with $E = 2.6 \times 10^4$ V m^{-1}.

spectrin dimer (a) and spectrin tetramer (b) solution. The extremum is more pronounced for the tetramer. From the decay curve plotted in fig. 3 on a logarithmic scale there is only one relaxation phenomenon. The relaxation time for the tetramer (7 μs) is significantly larger than that measured for the dimer. In the latter case it depends slightly on the electric field intensity (5 μs for $E = 1.4 \times 10^4$ V m^{-1} and 4 μs for $E = 2.6 \times 10^4$ V m^{-1}). The relaxation times do not vary with concentration, within the reproductibility of the determination which is about 10%.

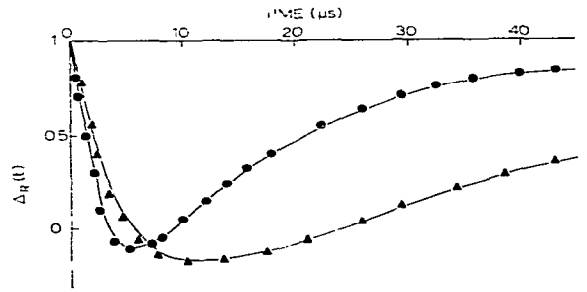

Fig. 4. Normalized birefringence (●, spectrin dimer; ▲, spectrin tetramer). The conditions are those of fig. 1 with $E = 2.6 \times 10^4$ V m^{-1}. The curves are drawn between the experimental points.

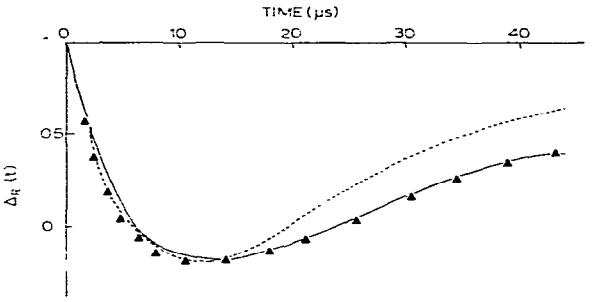

Fig. 5. Analysis of a fast reversing pulse experiment for spectrin tetramer. The points are experimental. The curves are computed according to eq. 6. (- - - - - -) Theoretical curve for a single relaxation time with $a_1 = 1$, $\tau_1 = 7$ μs, $r_1 = -25$. (———) Theoretical curve for two relaxations: $a_1 = 0.5$, $\tau_1 = 6$ μs, $r_1 = -10$, $a_2 = 0.5$, $\tau_2 = 11$ μs and $r_2 = -25$. $E = 2.6 \times 10^4$ V m^{-1}.

The normalized birefringence obtained for spectrin dimers and tetramers when a reversing pulse is applied is shown in fig. 4. It depends strongly on the state of polymerization of spectrin. We have tried to analyze these curves according to eq. 6. In the case of dimers and for the lowest electric field intensities, a satisfactory fit is obtained with a single relaxation phenomenon. This is no longer the case at higher electric field. For the tetramers two relaxation times are necessary to give a satisfactory fit with the experiments (fig. 5). Table 1 summarizes the values of the parameters

TABLE 1

Electro-optical parameters of spectrin dimers and tetramers determined from reversing pulse experiments

Parameters (a_i, τ_i and r_i) are obtained from reversing pulse experiments performed with an electric field intensity $E = 2.6 \times 10^4$ V m^{-1}. The rows 1 and 2 refer to the two relaxation processes. Eq. 6 is used; the fitting procedure is started with parameters obtained when only one relaxation phenomenon is considered. The fit is then improved by introducing a second relaxation mechanism. The same results are obtained from the build-up curves analyzed according to eq. 1.

Species	a_i	τ_i (μs)	r_i
Dimer 1	0.7	3.5	-45
2	0.3	7	+[illegible]
Tetramer 1	0.5	6	−10
2	0.5	11	−25

of eq. 6 computed for an applied electric field of 2.6×10^4 V m^{-1}. Under these conditions, the values of r_i are systematically opposite in intensity for spectrin dimers and tetramers.

4. Discussion

Branton and co-workers [11] have established the structure of the spectrin molecule by electron microscopy. The heterodimer appears as a flexible elongated structure of 1000 Å in length, showing the juxtaposition of several globular domains. The heterotetramers are formed by the head-to-head association of two dimers. Preparation of the samples for low-angle shadowing electron microscopy is somewhat drastic and it is worthwhile to consider if spectrin is solution has the same structure. Moreover, evidence for the elongated and peculiar flexible character of the spectrin molecule was obtained earlier from independent measurements [12].

The electric birefringence experiments of Mikkelsen and Elgsaeter [1] have already shown that the relaxation time of the spectrin dimer cannot correspond to a rigid rod-like structure of the size observed from electron microscopic experiments [11]. In agreement with this finding, we can show from the present study that using an elongation ratio $L/2b$ between 20 to 40 in eq. 5, the length of the spectrin dimer is between 650 and 750 Å.

The above-mentioned authors have also compared the properties of spectrin according to its state of polymerization but failed to observe any difference between the dimers of tetramers [2]. In similar experiments from the decay curve of birefringence obtained at lower electric field, we have found (fig. 3) some differences in the relaxation time of dimers and tetramers (from 4 to 7 μs) and shown that contrary to the findings of Mikkelsen and Elgsaeter [2], the solutions behave as a monodisperse material. The reason for this difference cannot reasonably be attributed to the nature of the samples as the spectrin solutions have been prepared in both cases under roughly similar conditions. We have also carefully ensured in the present work that no contaminant aggregates are present in the sample of spectrin tetramers (see section 2). However, we have used much lower electric fields than those of Mikkelsen and Elgsaeter [2]. It is possible that high electric fields can distort the shape of the spectrin molecule and introduce other relaxation processes than those we have studied in this work. In this respect, we noticed that the relaxation time of spectrin dimers was significantly smaller when the electric field intensity was increased from 1.4×10^4 to 2.6×10^4 V m^{-1}.

The small increase observed in the relaxation time of spectrin on association to tetramers was expected from the theoretical studies of Yu and Stockmayer [13] performed on a molecular model which mimicked approximatively the shape of the spectrin tetramer, i.e., two long rigid rods of equal length connected by a flexible joint.

Differences between spectrin dimers and tetramers are further emphasized when the samples are submitted to fast reversing electric pulses (fig. 2 and 4). The values of τ computed from the time t_m are consistent with those obtained from the birefringence decay (fig. 3). This experiment demonstrates further that spectrin carries a strong permanent dipole. It shows also that the relaxation phenomena observed are more complex processes, implicating at least two relaxation constants (table 1). In view of the very elongated structure of spectrin, it is possible that several local permanent dipole moments located at different points of the molecule contribute to the birefringence in reversing pulse experiments. A striking difference between spectrin dimers and tetramers appears in the values of $r_i = \mu_i^2/\Delta\alpha_i kT$ which are opposite in sign (table 1). This indicates that the apparent anisotropy of polarizability $\Delta\alpha$ is considerably modified as a consequence of the head-to-head association of two heterodimers. At present, it is not possible to determine the origin of this effect and to decide unequivocally if it is solely due to the increase in the length of the protein or whether it is more specifically caused by changes in the conformation of the peptide chains in the regions of the hinge linking the spectrin dimers or elsewhere in the molecule.

Acknowledgements

We wish to thank Professor M. Hanss with whom this work was initiated and Ms. A. Royer for her efficient technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (E.R. 157), the Délégation Générale à la Recherche Scientifique et Technique and the Institut National de al Santé et de la Recherche Médicale.

References

1 A. Mikkelsen and A. Elgsaeter, Biochim. Biophys. Acta 536 (1978) 245.
2 A. Mikkelsen and A. Elgsaeter, Biochim. Biophys. Acta 668 (1981) 74.
3 I. Tinoco and K. Yamaoka, J. Phys. Chem. 63 (1959) 423.
4 J.C. Bernengo, D. Herbage and B. Roux, Ber. Bunsenges. Phys. Chem. 80 (1976) 246.
5 J.T. Dodge, C. Mitchell and D.J. Hanahan, Arch. Biochem. Biophys. 100 (1969) 119.
6 M. Clarke, Biochem. Biophys. Res. Commun. 45 (1971) 1063.
7 E. Ungewickel and W. Gratzer, Eur. J. Biochem. 88 (1978) 379.
8 E. Fredericq and C. Houssier, Electric dichroism and electric birefringence (Clarendon Press, Oxford, 1973).
9 J.C. Bernengo, B. Roux and D. Herbage, Biopolymers 13 (1974) 641.
10 B. Roux, J.C. Bernengo, C. Marion and M. Hanss, J. Colloid Interface Sci. 66 (1978) 421.
11 D.M. Shotton, B.E. Burke and D. Branton, J. Mol. Biol. 131 (1979) 303.
12 Y. Lemaigre-Dubreuil, Y. Henry and R. Cassoly, FEBS Lett. 113 (1980) 231.
13 H. Yu and W.H. Stockmayer, J. Chem. Phys. 47 (1967) 1369.