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Electrooptical analysis of α-chymotrypsin at physiological salt concentration

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Abstract

The electric dichroism of α-chymotrypsin has been measured in a buffer containing 0.1 M Na^+, 10 mM Mg^{2+} and 25 mM Tris–cacodylate pH 7.2. The reduced dichroism as a function of the electric field strength can be represented by the orientation function for permanent dipoles and is not consistent with the orientation function for induced dipoles. After correction for the internal directing field, the dipole moment is 1.1×10^{-27} Cm (±10%), corresponding to 340 D, at 20°C. The assignment of the permanent dipole moment is confirmed by the shape of the dichroism rise curves, which require two exponentials with amplitudes of opposite sign for fitting. The dichroism decay time constants measured in the range of temperatures between 2 and 30°C indicate a temperature induced change of the structure, which is equivalent to an increase of the hydrodynamic radius from $r = 26.6$ Å at 2°C to 28.5 Å at 30°C. Our results demonstrate that electrooptical investigations of proteins with a high time resolution can be extended to physiological salt concentrations without serious problems by use of appropriate instruments.

Keywords: Electric dichroism; Dipole moment; Rotational relaxation; Reduced dichroism

1. Introduction

The spatial distribution of charged residues in many biological macromolecules is not symmetric and, thus, these macromolecules are associated with a dipole moment. Due to the large number of charges and due to the large dimensions of these molecules, the dipole moments can be extremely high. Dipole moments of macromolecules have been characterized experimentally by measurements of the response of their solutions to external electric fields. Most data reported in the literature have been obtained by investigations of the dielectric relaxation [1–4]. In this approach dipole moments are evaluated from the frequency dependence of dielectric constants. In order to get experimental data at a sufficient sensitivity, dielectric measurements are usually performed in the absence of buffer and/or salt. The analysis of biological macromolecules with a high charge density in salt-free solutions involves the risk that their conformation is not native. Furthermore, dielectric measurements require rather high concentrations of the material under investigation. Finally, dielectric investigations do not provide sufficient information on the nature of the dipole moment: the induced or saturating induced dipole moment of DNA double

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helices, for example, could not be distinguished from permanent dipole moments corresponding to an inherent anisotropy of the charge distribution [5–7].

Most of the problems associated with dielectric measurements can be avoided by electrooptical investigations [8,9], where the degree of molecular alignment induced by external electric fields is characterized by measurements of the optical anisotropy. Measurements of the optical anisotropy as a function of the field strength can be used for the quantitative determination of the dipole moment and of the limiting value of the optical anisotropy [10]. The combination of the optical anisotropy with the dipole moment is useful for comparison with the results of calculations from crystal structures, because the optical anisotropy provides information about the direction of the dipole vector. This information is not accessible from dielectric measurements. The concentration of the investigated molecular species required for measurements of the electric dichroism is given by the magnitude of the extinction coefficient and, thus, in most cases the concentration can be very low. An essential source of information in the electrooptical characterization of dipoles are the transients observed upon application of electric field pulses: the shape of these 'rise curves' provides valuable information for the assignment of the nature of dipole moments [11–14]. The same type of information may be obtained by reverse pulse experiments, but generators with reversal of the field vector in time intervals of a few ns, which are required for analysis of relatively small macromolecules, have not been constructed yet. Usually electrooptical measurements can be performed without serious problems at buffer or salt concentrations up to a few mM: the application range depends on the quality of the pulse generator and on the magnitudes of the electrical and of the optical anisotropies of the molecules under investigation. Some investigations of proteins were conducted in this laboratory up to salt concentrations of 10–20 mM [14–17]. Because biological macromolecules are polyelectrolytes with a potential high sensitivity of their conformation on the salt concentration, an instrument has been constructed for measurements at still higher salt concentrations corresponding to physiological conditions [18]. In the present investigation this instrument has been used for a characterization of the electrooptical parameters of α-chymotrypsin. We have selected α-chymotrypsin as a test case, because this protein has been analyzed previously by electrooptical procedures at low to medium salt concentrations [16]. The electrooptical parameters of this protein have been calculated from the coordinates of the crystal structure [16].

2. Materials and methods

Samples of α-chymotrypsin were obtained from Serva (Heidelberg, Germany) and from Sigma (Deisenhofen, Germany). The samples were dissolved in the standard buffer containing 0.1 M $NaClO_4$, 10 mM $Mg(ClO_4)_2$ and 25 mM Tris–cacodylic acid pH 7.2. Concentrations were determined by absorbance measurements using an extinction coefficient of 4.42×10^4 M^{-1} cm^{-1} at 282 nm.

The electric dichroism was measured by an instrument using the discharge of a 20 m cable for field pulse generation [18]. The load impedance was matched to the characteristic impedance of the cable by a compensation cell, which was flushed continuously by an electrolyte solution with an appropriate conductivity. The instrument has been used in an automatic mode under the control of a personal computer as described [18] with the following replacements: a Schoeffel GM 250 monochromator was used instead of a Bausch & Lomb high intensity monochromator; a Tektronix DSA 602 digitizing signal analyzer was used for transient storage of the electrooptical signals. The data were collected on a personal computer and were transferred to the facilities of the Gesellschaft für wissenschaftliche Datenverarbeitung mbH, Göttingen, for evaluation. Transients were evaluated by an efficient deconvolution procedure [19] using reference curves measured as birefringence signals of the buffer under conditions exactly corresponding to those used in the dichroism measurements.

3. Results

3.1. Stationary values of the dichroism and dipole moments

Electric field pulses applied to solutions of α-chymotrypsin led to a decrease in the intensity of

light transmitted by these solutions, when the plane of polarization was parallel to the field vector. Corresponding experiments with the plane of polarization at the magic angle of 54.74° did not reveal changes of the transmitted light. Thus, electric field pulses induced orientation of α-chymotrypsin and did not induce conformation changes. In this respect the observed effects could be analyzed without complications. However, due to the high salt concentrations used in the present investigation, the electric field pulses did not only induce orientation effects, but also an increase of the temperature in the solution. In order to limit this temperature jump effect, the length of the field pulses applied by the instrument (determined by the length of the cable) was restricted to 200 ns.

Because of the relatively short electric field pulses, usually the stationary values of the optical signals could not be determined directly at a sufficient accuracy. Thus, the stationary values of the electric dichroism have been determined by exponential fitting of the dichroism rise curves. Some details of the fitting procedure are discussed below.

The stationary values of the reduced dichroism have been analyzed in terms of the orientation functions for permanent and induced dipoles. As shown in Fig. 1, the experimental data can be fitted at a high accuracy to the orientation function for permanent dipoles and are not consistent with the orientation function for induced dipoles. The dipole moments and the limit values of the electric dichroism obtained from these fits are compiled in Table 1.

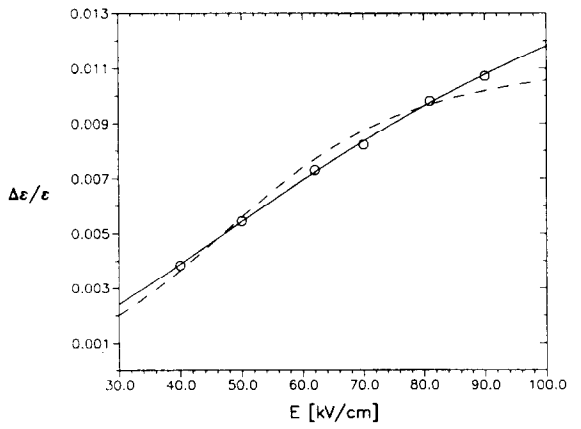

Fig. 1. Stationary dichroism of α-chymotrypsin ($\Delta\epsilon/\epsilon$) at different electric field strengths E at 10°C. The solid line represents a least squares fit of the data according to the orientation function for permanent dipoles with $\mu_{exp} = 1.6 \times 10^{-27}$ Cm and $(\Delta\epsilon/\epsilon)_\infty = 0.028$ (± 0.006). The dashed line represents a least squares fit according to the induced dipole orientation function.

Table 1
Electrooptical data of α-chymotrypsin obtained by least squares fits of stationary values of the reduced dichroism at different electric field strengths to the orientation function of permanent dipoles

T [°C]	$(\Delta\epsilon/\epsilon)_\infty$ -	μ_{exp} [10^{-27} Cm]	μ_{corr} [10^{-27} Cm]
2	0.02	1.8	1.2
10	0.03	1.5	1.0
20	0.02	1.7	1.1
30	0.03	1.3	0.87

Estimated accuracies: dipole moments ±10%, $(\Delta\epsilon/\epsilon)_\infty$ ±20%

Experimental values of dipole moments have to be corrected for the difference between the external applied electric field strength E_e and the internal directing field strength E_i. The shape of α-chymotrypsin is almost spherical and, thus, the correction factor has been calculated according to the standard formula for spheres [20]:

$$E_i = [3\epsilon/(2\epsilon + 1)]\, E_e \qquad (1)$$

where ϵ is the dielectric constant of the solvent. The corrected values of the dipole moments are in the range of 260 to 350 D (cf. Table 1). These values are somewhat lower than those obtained previously at lower salt concentrations.

3.2. Dichroism transients and time constants of rotational diffusion

The dichroism decay curves measured after pulse termination could be fitted by single exponentials at a high accuracy (cf. Fig. 2). Because of the temperature jump induced by the electric field pulses, the dichroism decay time constants obtained after pulses of different field strengths are measured at somewhat different temperatures, even when the pulses are applied at the same starting temperature. The change of the temperature induced by the highest field pulses is ca. 2°C (in the standard buffer). The magnitude of the temperature jump can be easily calculated and has also been checked by a simple chemical indica-

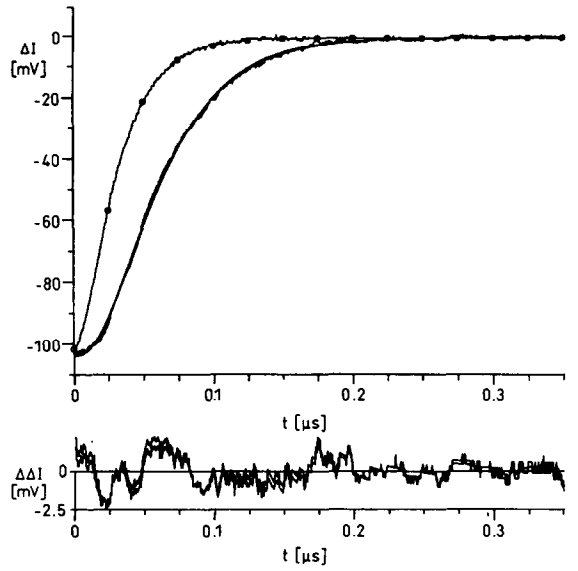

Fig. 2. Dichroism decay of α-chymotrypsin after an electric field pulse of 90 kV/cm at 2°C; average of 26 shots (continuous line with noise); protein concentration: 23.1 μM. The data marked by circles show the reference used for deconvolution (obtained by birefringence detection upon application of a 90 kV/cm pulse to the buffer; amplitude normalized to that of the dichroism decay). The continuous line without noise, which can hardly be distinguished from the dichroism decay curve, represents a least squares fit by a single exponential with $\tau = 34$ ns. The lower panel shows the residuals ($\Delta\Delta I$) for fits with one and two exponentials; the residuals for the fit by one exponential are only slightly larger than those for the fit by two exponentials.

tion system. All the rotational relaxation time constants determined in the present investigation have been corrected for the temperature jump effect by the standard viscosity/temperature conversion factor to the temperature before application of the field pulse:

$$\tau_d^0 = \left[(\eta^0/T^0)/(\eta^1/T^1)\right]\tau_d^1 \qquad (2)$$

where η^0 and η^1 are the viscosities at the temperatures T^0 before the field pulse and T^1 after the field pulse, respectively; the temperatures T^0 and T^1 are given in Kelvin degrees; τ_d^0 and τ_d^1 are the time constants at the temperatures T^0 and T^1, respectively. The decay times obtained after pulses of different field strengths are constant after correction (cf. Fig. 3). Analysis of a large number of experimental data sets did not show any reproducible dependence of the corrected τ_d-values on the electric field strength.

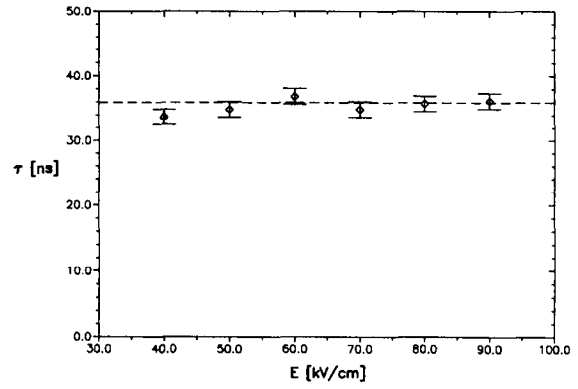

Fig. 3. Time constants τ_d obtained from the dichroism decay of α-chymotrypsin measured after pulses of different field strengths E. The τ_d-values have been corrected to the temperature before pulse application (10°C) according to Eq. (2). The dashed line corresponds to an average of the time constants weighted according to the amplitude of the dichroism decay. Concentration of α-chymotrypsin 23.1 μM.

The decay time constants have been measured over a wide range of concentrations. The complete absence of a concentration dependence (cf. Fig. 4) demonstrates that there is no aggregation under our experimental conditions. Decay time constants measured at different temperatures reveal a remarkable dependence, when the values are transformed to the same reference state of 2°C (cf. Table 2): the transformed time constants increase continuously with increasing temperature. Because the shape of α-

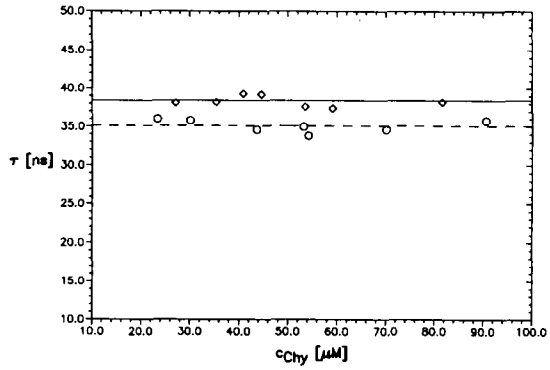

Fig. 4. Dichroism decay time constants τ_d of α-chymotrypsin measured at different protein concentrations (c) and at two different temperatures: (○) 10°C; (◇) 20°C; all time constants have been transformed to 2°C by the standard viscosity/temperature conversion factor.

Table 2
Dichroism decay time constants, effective hydrodynamic volumes and effective hydrodynamic radius of α-chymotrypsin at different temperatures

T [°C]	τ_d (T) [ns]	τ_d (2°C) [ns]	V_h [10^4 Å^3]	r_h [Å]
2	34.6	34.6 ± 1.2	7.87	26.6
10	26.7	35.1 ± 1.2	7.99	26.7
20	21.6	38.4 ± 0.9	8.72	27.5
30	18.5	42.6 ± 2.6	9.71	28.5

The time constants are averages from different independent series of measurements; in each series the individual values measured after pulses of different field strengths were weighted according to the dichroism amplitudes; the accuracies given in the third column of the table have been estimated from the variation of the results.

chymotrypsin is almost spherical, the time constants τ_d may be converted into effective hydrodynamic volumes V_h according to the equation for spheres:

$$V_h = \tau_d \cdot kT/\eta \quad (3)$$

where η is the solvent viscosity at the absolute temperature T and k is the Boltzmann constant. We have also included in Table 2 the effective hydrodynamic radius r_h, which indicates an increase of the protein dimensions with the temperature. The existence of some change of the protein conformation at increasing temperature was also indicated by a change of the sensitivity to UV irradiation: at 30°C the protein was more sensitive to the light used for the measurements than at lower temperatures. For this reason the irradiation times had to kept at the absolute minimum at 30°C.

The electrooptical transients induced upon application of electric field pulses have been analyzed by the same algorithms as the decay curves. As shown in Fig. 5, the dichroism rise curves require 2 exponentials for fitting. The amplitudes associated with these exponentials are of opposite sign. Sign and magnitudes of these amplitudes are characteristic of relaxation curves starting with a zero slope, which indicate the existence of a permanent dipole moment or of a slow polarizability. The experimental rise curves are not consistent with a simple induced dipole associated with a fast polarizability. The risetime constants τ_2 measured at different electric field strengths E can be represented approximately by a linear relation (cf. Fig. 6), which extrapolates to a τ_2-value at $E = 0$, which is much larger than the

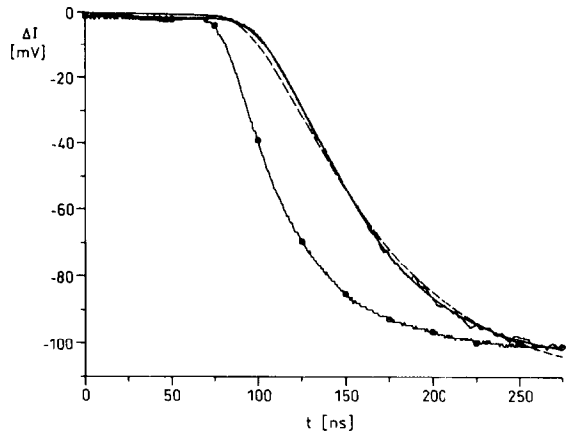

Fig. 5. Dichroism rise curve of α-chymotrypsin induced by an electric field pulse of 90 kV/cm at 30°C. The dashed line shows a least squares fit by a single exponential; the fit by two exponentials with amplitudes of opposite sign can hardly be distinguished from the experimental curve ($\tau_1 = 13$ ns, $\tau_2 = 27$ ns, $A_1 = -97$ mV, $A_2 = 271$ mV); the data marked by circles represent the reference used for deconvolution, obtained by birefringence detection upon application of a 90 kV/cm pulse to the buffer.

dichroism decay time constant measured at the same temperature. This result again supports the assignment of a permanent dipole moment, although the exact relation of the risetime constants to the decay time constants expected according to the equation of Benoit [21] cannot be verified in the present case, because of a limited accuracy and the extrapolation over a too wide range of field strengths.

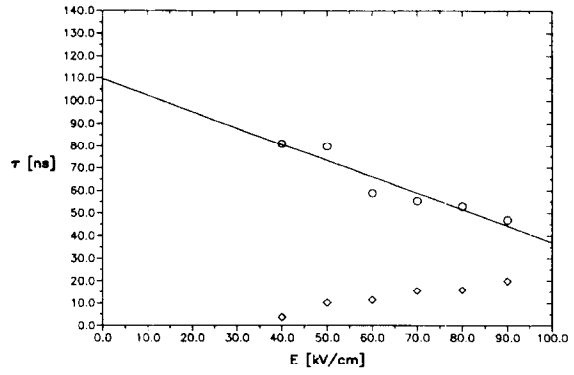

Fig. 6. Dichroism risetime constants of α-chymotrypsin τ_1 (◇) and τ_2 (○) at different electric field strengths E. The straight line shows a linear regression of $\tau_2 = f(E)$.

4. Discussion

Dipole moments of many proteins have been measured already more than 50 years ago [1], mainly by the dielectric relaxation technique, but the nature of these dipole moments remained unclear until recently. The techniques used for the characterization of protein dipoles were not sufficient to distinguish between various cases like proton fluctuations [22], polarization of ion atmospheres [23–25] and standard permanent dipoles corresponding to an anisotropy of the charge distribution at equilibrium in the absence of an external electric field. With respect to the experimental characterization, progress has been made by application of electrooptical techniques with ns time resolution: it has been possible to characterize the time constants of ion polarization [13] and to demonstrate at a sufficient time resolution that the dichroism rise curves of several proteins [14–17] are consistent with the existence of permanent dipoles. The experimental results have been complemented by calculation of dipole moments based on crystal structures [16,17,26]. The agreement of the results obtained by these procedures support the validity of the major conclusions.

Charged residues are known to be located almost exclusively at the surface of proteins. It must be expected that these residues are shielded by increasing concentrations of ions. As shown by the data compiled in Table 3, this expectation is verified: the dipole moment of α-chymotrypsin obtained in the present investigation is smaller than the values obtained previously at lower salt concentrations. However, it is remarkable that the decrease of the dipole from low to physiological salt concentrations remains relatively small. Thus, only a rather small amount of charged protein residues is shielded at 125 mM monovalent + 10 mM bivalent salt concentration. According to the available data, the limit value of the electric dichroism is more strongly affected by addition of Mg^{2+}-ions than by addition of monovalent ions. This effect may be explained by preferential binding of Mg^{2+} to some sites of the protein. Because the electric dichroism of α-chymotrypsin is close to zero, its value can be affected already by relatively small changes of the direction of the dipole vector. Binding of Mg^{2+}-ions to a single protein site may be sufficient to explain the observed dependence of the dichroism on the Mg^{2+}-concentration.

The dipole moment and the limiting reduced dichroism obtained from the present measurements are rather close to the results of calculations published previously [16]. The values calculated for α-chymotrypsin at pH 7.0 are 1.55×10^{-27} Cm for the dipole moment and 0.23 for the limiting reduced dichroism. Thus, the calculated values are larger than the measured ones, but the deviations are not too large in view of the approximations involved in the calculations. One of the problems is an appropriate treatment of the shielding of protein charges by ions in solution.

According to the theory [27], the dichroism decay of molecules without symmetry elements is composed of 5 exponentials. In practice it is virtually impossible to resolve these exponentials. Usually single exponentials are sufficient for fitting of dichroism decay curves. As shown in a recent investigation of different hemoglobin species [17], the observed 'apparent' relaxation time constant may be changed considerably, even if the dimensions of the

Table 3
Compilation of electrooptical data of α-chymotrypsin obtained at 20°C at different salt concentrations. The data for the highest salt concentration are from the present investigation, the other data are from Ref. [16]

c_+ [mM]	c_{Mg} [mM]	pH -	$(\Delta\epsilon/\epsilon)_z$ -	μ_{corr} [10^{-27} Cm]	μ_{corr} [Da]
2.4	-	7.0	0.11	1.4	420
7.4	-	7.0	0.13	1.4	420
12.4	-	7.0	0.13	1.4	420
9	0.2	8.3	0.10	1.5	450
9	1	8.3	0.08	1.5	450
19	1	8.3	0.05	1.7	510
125	10	7.2	0.02	1.1	340

Estimated accuracies: dipole moments ± 10%, $(\Delta\epsilon/\epsilon)_z$ ± 20%.

molecule remain virtually constant. In the case of hemoglobin species, the 'apparent' time constants were changed by a variation of the relative orientation of the electric dipole vector with respect to the preferential axis of light absorption, which resulted in a change of the amplitudes. Although the individual time constants of the dichroism decay remained unchanged, the change of the amplitudes led to a considerable change of the resulting overall 'apparent' decay time constants. Apparent 'acceleration' or 'retardation' effects of the dichroism decay may be particularly large, when the stationary dichroism is close to zero, which appears to be very common among proteins.

The experimental dichroism decay time constants observed for α-chymotrypsin show a remarkable dependence on the temperature, which clearly indicates a temperature induced change of the protein conformation. It remains to be established, whether these results are due to changes of the overall protein dimensions or due to some change in the direction of the dipole vector with respect to the preferential axis of light absorption. Potential 'acceleration' or 'retardation' effects in the dichroism decay of α-chymotrypsin have to be considered, because its stationary dichroism is close to zero. However, in the present case there is experimental evidence against a strong influence of these special effects. Measurements of the stationary dichroism at different temperatures did not reveal any variation of the limiting value of the reduced dichroism (cf. Table 1) and, thus, the relative orientation of the dipole vector to the preferential axis of light absorbance remains constant. Under these conditions the changes of the dichroism decay time constants are mainly assigned to changes of the overall protein dimensions. As shown by the data compiled in Table 2, the increase of the protein dimensions with increasing temperature is remarkably large: the effective radius of the protein changes from 26.6 Å at 2°C to 28.5 Å at 30°C. We are not aware of any similar investigations of α-chymotrypsin or of any other proteins.

Our present analysis was started as a test case for the feasibility of electrooptical investigations at physiological salt concentrations by an instrument developed recently [18]. The results are encouraging, because data of sufficient accuracy have been obtained for a relatively small protein, even though its dichroism is rather small. Although electrooptical data cannot be used to determine structures at atomic resolution, the electrooptical approach is at least useful as a complement to other techniques for investigation of structures and their dynamics in solution. We conclude that electrooptical investigations of proteins are not restricted to low salt concentrations, but can be extended to physiological salt concentrations without serious problems by application of appropriate experimental techniques.

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References

[1] J.J. Oncley, in J.J. Cohn and J.T. Edsall (Editors), Proteins, Amino Acids and Peptides, Am. Chem. Soc. Monograph Series (1943).
[2] E.H. Grant, R.J. Sheppard and G.P. South, Dielectric Behaviour of Biological Molecules in Solution, Clarendon Press, Oxford, 1978.
[3] N.A. Hill, W.E. Vaughan, A.H. Price and M. Davies, Dielectric Properties and Molecular Behaviour, Van Nostrand, London, 1969.
[4] S. Takashima, Electrical Properties of Biopolymers and Membranes, Adam Hilger, Philadelphia, 1989.
[5] S. Takashima, J. Mol. Biol., 7 (1963) 455.
[6] S. Takashima, C. Gabriel, R.J. Sheppard and E.H. Grant, Biophys. J., 46 (1984) 29.
[7] B. Saif, R.K. Mohr, C.J. Montrose and T.A. Litowitz, Bipolymers, 31 (1991) 1171.
[8] E. Fredericq and C. Houssier, Electric Dichroism and Electric Birefringence, Clarendon Press, Oxford, 1973.
[9] C.T. O'Konski (Editor), Molecular Electrooptics, Part 1: Theory and Methods, Marcel Dekker, New York, 1976; Part 2: Applications to Biopolymers, M. Dekker, New York, 1978.
[10] C.T. O'Konski, K. Yoshioka and W.H. Orttung, J. Phys. Chem., 63 (1959) 1558.
[11] I. Tinoco Jr., J. Am. Chem. Soc., 77 (1955) 4486.
[12] I. Tinoco Jr. and K. Yamaoka, J. Phys. Chem., 63 (1959) 423.
[13] D. Porschke, Biophys. Chem., 22 (1985) 237.

[14] D. Porschke, Biophys. Chem., 28 (1987) 137.
[15] D. Porschke, K. Tovar and J. Antosiewicz, Biochemistry, 27 (1988) 4674.
[16] J. Antosiewicz and D. Porschke, Biochemistry, 28 (1989) 10072.
[17] J. Antosiewicz and D. Porschke, Biophys. J., 68 (1995) 655.
[18] D. Porschke and A. Obst, Rev. Sci. Instr., 62 (1991) 818.
[19] D. Porschke and M. Jung, J. Biomol. Struct. Dynam., 2 (1985) 1173.
[20] C.J.F. Bottcher, Theory of Electric Polarization, Vol. 1, Elsevier, Amsterdam, 1973.
[21] H. Benoit, Ann. Phys., 6 (1951) 561.
[22] J.G. Kirkwood and J.B. Shumaker, Proc. Natl. Acad. Sci. USA, 38 (1952) 855.
[23] W. Scheider, Biophys. J., 5 (1965) 617.
[24] R. Lumry and R.H. Yue, J. Phys. Chem., 69 (1965) 1162.
[25] G. Schwarz, J. Phys. Chem., 66 (1962) 2636.
[26] S. Takashima and K. Asami, Biopolymers, 33 (1993) 59.
[27] W.A. Wegener, R.M. Dowben and V.J. Koester, J. Chem. Phys., 70 (1979) 622.