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Ionic strength dependence of the electric dissociation field effect

Investigation of 2,6-dinitrophenol and application to the acid-alkaline transition of metmyoglobin and methemoglobin

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We present data on the ionic strength dependence of the dissociation field effect (2nd Wien effect) of the protolytic reaction of 2,6-dinitrophenol in aqueous solution, monitored through optical absorption changes in high electric fields. The results are in very good agreement with the Onsager-Liu theory. We then investigate the field strength dependence of the acid-alkaline transition, i.e., the hydrolysis reaction of the water coordinated to the heme iron, in metmyoglobin and methemoglobin. The true field effect of the reaction is determined from measurements in buffers which exhibit no field effect. We conclude that negative charges on the protein influence the field effect in methemoglobin but not in metmyoglobin. The Onsager-Liu theory is applied to estimate the number of charges involved.

1. Introduction

The effect of electric fields on chemical equilibria has been studied continuously since the discovery of the dissociation field effect by Wien and co-workers [1]. At present interest is being concentrated on field effects in biopolymer systems, since electric field-induced structure changes are a way of coupling electric field forces with biochemical reactions such as nerve excitation, ion transport through membrane protein channels and secretion of hormones from vesicles (for recent reviews on these topics see refs. 2 and 3).

Electric fields influence dipolar and ionic equilibria [4,5]. While the field effect on dipolar equilibria can be described with Gibbs's thermodynamic relations extended by electrostatic free energy terms, the field effect on ionic equilibria, i.e., the dissociation field effect or 2nd Wien effect, is understood in terms of Onsager's kinetic theory in which particle fluxes for ionic recombination and dissociation are considered separately [6].

Important questions remain to be clarified even for very simple types of ionic equilibria: How is the field effect influenced by the ionic strength of the medium, when the reactive site is shielded by an ionic atmosphere, and what is the effect of fixed charges in the neighborhood of the reactive site, for example, in zwitterions? An answer to these questions is of relevance if field effects on biopolymers are to be analysed more quantitatively.

A theory of the ionic strength dependence of the dissociation field effect has been developed in Onsager's group [7,8] but has not been tested experimentally so far.

In the present paper we report measurements

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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of the ionic strength dependence of the field effect on a reaction of type $A^- + H^+ = AH$, using 2,6-dinitrophenol in aqueous solution. Comparing the results with Onsager's extended theory, we find excellent agreement. We then apply the theory to field effect measurements of the acid-alkaline transition in methemoglobin and metmyoglobin in order to estimate the effective charges at the reactive site in the protein. The acid-alkaline transition of the heme proteins denotes the reaction $Fe^+H_2O + OH^- = FeOH + H_2O$, where a proton is transferred from the water molecule bound at the heme Fe(III) to an OH^- [9]. The reaction occurs in the microsecond time range and can be perturbed by high electric fields [10]. For a quantitative evaluation of the field dependence of the equilibrium constant it is necessary to investigate the reaction in buffers of known field dependence. Only by adding buffers can the unknown field effects of other protolytic groups on the protein be eliminated. On the other hand, the addition of buffers increases the ionic strength and necessitates for quantitative analysis an understanding of the ionic strength dependence of the dissociation field effect.

2. The extended Onsager theory *

The kinetic theory of the dissociation field effect [6], presented by Onsager in 1934, did not consider explicitly the effects of the ionic atmosphere. The expression obtained by Onsager (eq. 1) is only valid in the case of zero ionic strength or for finite ionic strength in the limit of high electric fields, where the ionic atmosphere is destroyed. In the linear approximation, which is sufficient in aqueous solutions, the field strength dependence of the equilibrium constant is given by

$$\frac{\lim K'(E)}{K(E=0)} = \frac{K(E)}{K(E=0)} = 1 + 2\beta q + \ldots \tag{1}$$

$$\frac{\lim K'(E)}{K'(E=0)} = 1 + 2\beta q - 2\kappa q + \ldots \tag{1a}$$

where K denotes the thermodynamic equilibrium constant, K' the concentration-equilibrium constant and E the electric field strength.

κ^{-1} denotes the Debye-Hückel radius of the ionic atmosphere, q is the Bjerrum radius

$$q = \frac{z_A z_B e^2}{2(4\pi\varepsilon_0)\varepsilon kT}$$

and β is given by

$$\beta = \frac{|z_A| u_A + |z_B| u_B}{2(u_A + u_B)kT} \cdot e|E|$$

z_A, z_B denote the charge numbers, u_A, u_B the mechanical mobilities of the reacting ions, and ε is the dielectric constant.

For a symmetrical electrolyte, i.e., $|z_A| = |z_B|$, or for an unsymmetrical electrolyte with greatly differing mobilities, i.e., $u_A \gg u_B$, the dependence on the transport quantities disappears and β reduces to

$$\beta = \frac{|z_A| e}{2kT} \cdot |E|$$

An outline of the perturbation method used to extend the theory to finite ionic strength is given by Onsager and Liu in ref. 7 and explicit expressions are derived by Liu in ref. 8. The extended theory yields for symmetrical electrolytes

$$\frac{K'(E)}{K(E=0)} = 1 + 2\beta q + 2\kappa q \cdot f(a) \tag{2}$$

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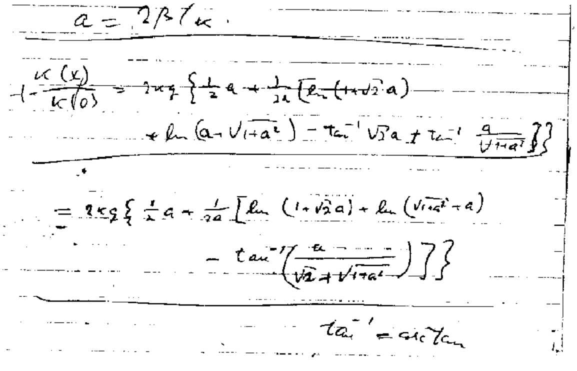
* Eq. 2 was presented by Onsager to Eigen's group at Göttingen during his 1968 sabbatical stay. Onsager's handwritten formula is reproduced below.



$$a = 2\beta/\kappa$$

$$-1 + \frac{K(x)}{K(0)} = 2\kappa q\left\{\frac{1}{2}a + \frac{1}{2a}\left[\ln(1+\sqrt{2}a) + \ln(a+\sqrt{1+a^2}) - \tan^{-1}\sqrt{2}a + \tan^{-1}\frac{a}{\sqrt{1+a^2}}\right]\right\}$$

$$= 2\kappa q\left\{\frac{1}{2}a + \frac{1}{2a}\left[\ln(1+\sqrt{2}a) + \ln(\sqrt{1+a^2}+a) - \tan^{-1}\left(\frac{a}{\sqrt{2}+\sqrt{1+a^2}}\right)\right]\right\}$$

$\tan^{-1} = \mathrm{arc\,tan}$

where the function $f(a)$ with $a = 2\beta/\kappa$ is given by

$$f(a) = \frac{1}{2a}\left\{\ln(1+\sqrt{2}a) + \ln(a+\sqrt{1+a^2}) - \arctan\frac{a}{\sqrt{2}+\sqrt{1+a^2}}\right\} \qquad (3)$$

For zero field strength, eq. 3 yields $f(a=0) = 1$ and eq. 2 reduces to the ionic strength dependence of the equilibrium constant according to the Debye-Hückel theory

$$\frac{K'(E=0)}{K(E=0)} = 1 + 2\kappa q + \ldots$$

At low field strength, i.e., $a \ll 1$, eq. 2 yields

$$\frac{K'(E)}{K(E=0)} = 1 + 2\kappa q + 3.542\frac{\beta^2 q}{\kappa} + \ldots$$

This weak-field approximation is also cited in ref. 11. At sufficiently high field strength $f(a \to \infty) = 0$ and the field dependence reaches asymptotically the limiting law eq. 1.

Eq. 2 is not yet quite correct because effects of the dissociation-recombination kinetics on the relaxation field of the ionic atmosphere are not included (S. Provencher, personal communication; cf. ref. 12).

In figs. 3 and 4 we have plotted the data in the form

$$\delta \ln K = \frac{K'(E) - K'(E=0)}{K(E=0)} = 2\beta q + 2\kappa q\left[f\left(\frac{2\beta}{\kappa}\right) - 1\right] \qquad (4)$$

It can be seen from fig. 3 that the screening of the ionic atmosphere is dominant at weak fields, leading to a quadratic field strength dependence which changes to a linear dependence at higher fields. However, already at an ionic strength of about 1 mM the asymptotic linear law is only reached at field strengths greater than 100 kV/cm which are hardly attainable in aqueous solution. The experimental and theoretical curves presented in fig. 3 also show that the field effect does not exhibit a very pronounced threshold behavior as may be inferred from eq. 1a if one assumes that a critical field, given by $\beta_0 = \kappa$ is necessary to destroy the ionic atmosphere before the field effect appears.

3. Materials and methods

The electric field pulse method with detection of optical absorbance closely follows the design of the apparatus developed at the Max-Planck-Institut für Biophysikalische Chemie at Göttingen [5,13,14]. The field pulses are generated by the discharge of a coaxial cable of about 300 m length. At variable times after the voltage is applied to the measuring cell by a spark gap switch, the cable can be short-circuited at the other end with a triggered spark gap and the energy is dissipated in an ohmic resistance equal to the cable impedance of 50 Ω. In each experiment the voltage time course is registered by means of a capacitance voltage divider on a Data Lab DL 905 transient recorder. Calibration is done with a Tektronix P 6015 high-voltage probe.

Fig. 1 shows the characteristic stepwise discharge of the cable, monitored with a small current viewing resistor in series with the measuring cell. The voltage drops occur every 3.4 μs and three to four voltage plateaus are used for evaluating the signals of the field effect at higher ionic strengths. The cable discharge method, exhibiting well-defined voltage steps, proves to be particularly well suited to obtain directly the derivative $\partial A/\partial E$ (A, absorbance; E, electric field strength).

The optical arrangement is equipped with a Glan-Thomson polarizer in front of the cell, in order to separate chemical and electrodichroic effects due to orientation of the molecules [15]. An RCA 1P28 photomultiplier is operated with one or two dynodes, and its power supply (Spellmann) is driven from d.c.-voltage sources to avoid electrical perturbations. The stationary photocurrent is measured by an I/U converter and the photosignals proceed through an a.c.-coupled emitter-follower to a broad-band amplifier (Keithley). The signals are stored in a Biomation 6500 transient recorder (6 bit, min. 2 ns per point). Calibration is done by applying defined current pulses to the photomultiplier load resistance.

The measuring cell into which the light is

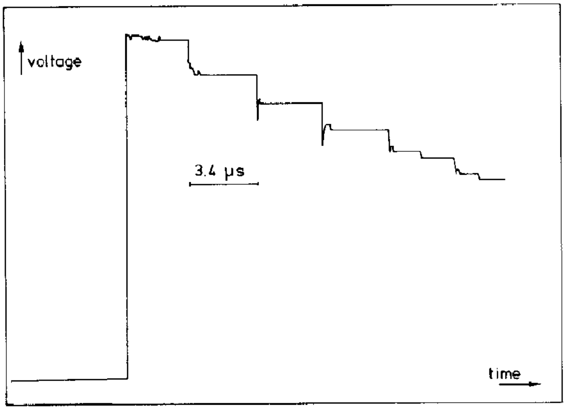


Fig. 1. Electric field pulse method. Voltage across the cell during cable discharge; the cell resistance is approx. 1 kΩ.

focussed has conical quartz windows and contains the solution between two platinum electrodes separated by a distance of 0.50 cm. The time resolution of the apparatus has been tested by monitoring the Kerr effect of urea. With a photomultiplier load resistance of 1 kΩ a time constant of 20–30 ns is found. The measurements on the dissociation field effects are usually performed with a 6.8 kΩ load, yielding a time constant of 150 ns.

2,6-Dinitrophenol was purchased from Fluka. Sperm whale myoglobin was a freeze-dried product from Serva. Stock solutions were purified from denatured material by centrifugation and membrane filtration. Human hemoglobin was prepared from bloodbank samples [16]; the concentrated hemoglobin solution was poured into liquid nitrogen and stored as small droplets at −60 °C. Oxidation to methemoglobin was performed with potassium hexacyanoferrate(III) and subsequent purification using a Sephadex G-25 column.

4. Results

4.1. 2,6-Dinitrophenol

2,6-Dinitrophenol is an acid with a pK value of 3.70. Spectrophotometric titration of the indicator yielded the same value as given in literature [17,18]. The protolytic reaction equilibrates very fast, with a time constant $\lesssim 150$ ns at pH 4, i.e., at about the time resolution of the optical detection system under the conditions used. No significant dependence of the optical signal on the position of the polarizer relative to the field direction is found.

Two series of measurements have been performed: (i) field strength dependence of the absorption changes at constant ionic strength (2 mM KCl); (ii) ionic strength dependence at constant field strengths. For the latter case, the cable voltages have been chosen such that the desired voltage across the cell lies within the first three or four voltage steps of the cable discharge, thus allowing reliable interpolation.

Fig. 2 shows examples of the optical signals as a function of time. When the electric field is applied to the solution, the light intensity at a wavelength of 430 nm decreases, indicating that the concentration of the deprotonated form of the acid is increased. The optical absorption changes follow the time course of the electric field without measurable delay; at the voltage steps which occur every 3.4 μs one observes the corresponding steps in the optical absorbance.

It is also seen that the temperature dependence of the protolytic reaction contributes to the absorption change – already in solution without added salt. Separation of electric field and temperature effects can be easily achieved: during the travelling time of the wave in the cable there is a constant voltage across the cell (see fig. 1). The temperature therefore rises linearly with time and the field effect can be obtained by linear extrapolation to the time of the voltage steps.

The difference of the baseline levels before and after the field pulse is caused by the net temperature change. This effect becomes dominant at higher ionic strengths and restricts evaluation of the field effect at values greater than about 0.01 M. Moreover, perturbations are observed at these conductivities which begin a few microseconds after the field is applied and are possibly due to pressure effects of the shock wave generated.

The relative change of the equilibrium constant according to eq. 4 is calculated from

$$\delta \ln K = \frac{\delta I}{I} \cdot \frac{1}{2.3 \Delta \varepsilon d} \left(\frac{1}{c_{\mathrm{AH}}} + \frac{1}{c_{\mathrm{H}}} + \frac{1}{c_{\mathrm{A}}} \right) \cdot \frac{1}{f_{\pm}^{2}} \tag{5}$$

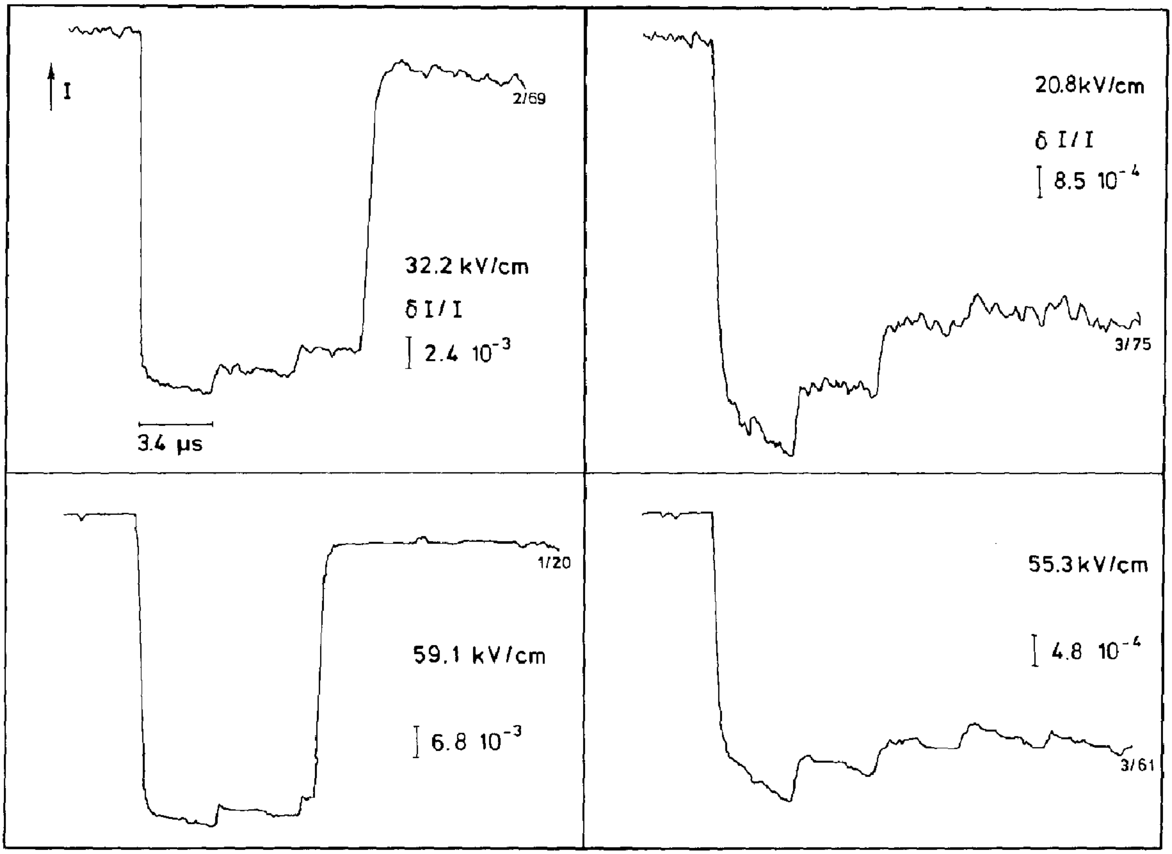


Fig. 2. Dissociation field effect of 2,6-dinitrophenol in aqueous solution. Change of light intensity vs. time at the field strengths indicated. Concentration of acid 0.145 mM; pH 3.55, 20 ° C, wavelength 430 nm; (left panels) without added salt, (right panels) with 2 mM KCl; there is no short-circuit of the cable discharge in the right-hand panels.

with $\Delta\varepsilon = 7900\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$, the difference of the extinction coefficients of the deprotonated (A) and protonated (AH) forms at 430 nm; $d = 1.95$ cm, optical light path (large cell); c_i, the concentrations of A^-, H^+ and AH, determined from the total concentration and the pH value measured; $\delta I/I$, the relative change of light intensity; and $f_{\pm}^2$ the product of activity coefficients (Debye-Hückel values are used).

The experimental results together with the theoretical curves according to eq. 4 are shown in figs. 3 and 4.

4.2. Azo dyes

Preliminary experiments have been performed with the dyes dimethyl yellow, methyl orange, and methyl red in aqueous solutions, in order to probe the influence of charged groups which are at defined distances from the reactive site, i.e., the proton-binding group. Dimethyl yellow, an uncharged base with $pK = 3.25$ [17] should exhibit no field effect. Methyl orange ($pK = 3.45$) and methyl red ($pK = 5.0$) are zwitterions in the protonated form and a field effect is to be expected.

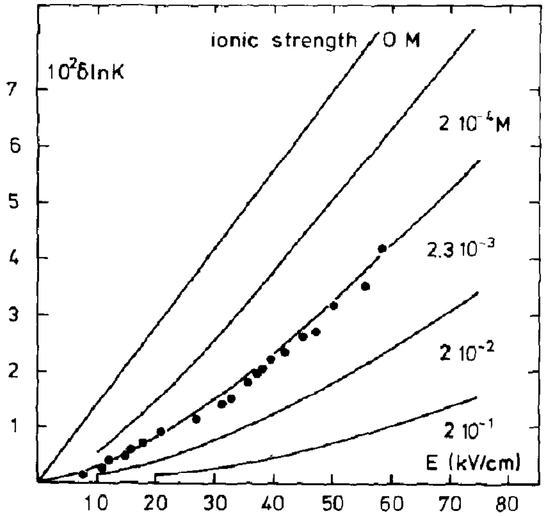


Fig. 3. Dissociation field effect of 2,6-dinitrophenol in aqueous solution. Field strength dependence of the equilibrium constant. Experimental points: concentration of acid 0.145 mM, pH 3.55, 20 °C, 2 mM KCl. (———) Theoretical dependence according to eq. 4 at different ionic strengths.

We could observe no chemical field effect with dimethyl yellow and methyl orange, electrodichroic effects, however, do appear.

Methyl red shows a pronounced chemical field effect with a relaxation time of 0.65 μs at a concentration of 38 μm and pH 4.80, yielding a diffusion-controlled recombination rate constant of 4×10^{10} M^{-1} s^{-1}. The field effects depend slightly on the angle of light polarization with respect to the electric field, indicating that more complex structures may be present. For light polarized parallel (//) and perpendicular ($\perp$) to the field, one obtains at $E = 77$ kV/cm; $\delta \ln K(//) = 0.068$ and $\delta \ln K(\perp) = 0.056$.

We interpret the result that methyl orange shows no field effect whereas methyl red does, which is about half of the theoretical value for a 1 : 1 electrolyte ($(\delta \ln K(//) + 2\delta \ln K(\perp))/3 = 0.06$ at 77 kV/cm), as being due to the different positions of the negative group. While in methyl red the negatively charged carboxylic group in the *ortho* position is very close to the proton-binding nitrogen, the negatively charged sulfonate group in methyl orange is in the *para* position to the azo group.

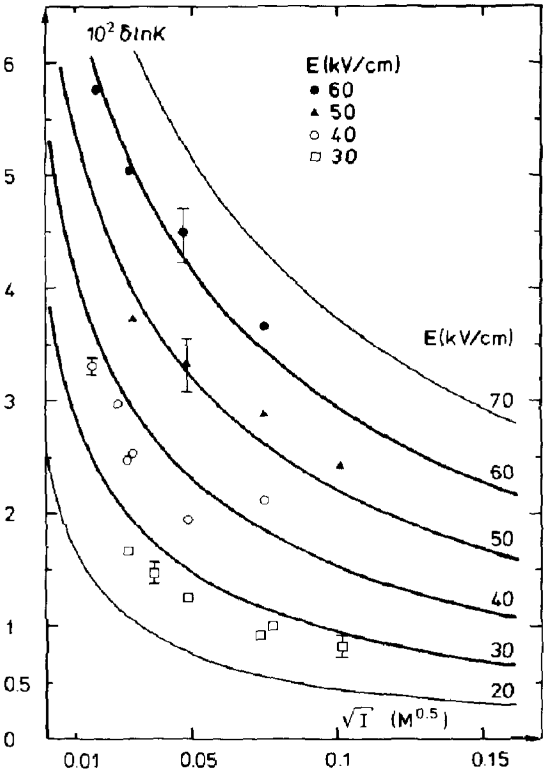


Fig. 4. Dissociation field effect of 2,6-dinitrophenol in aqueous solution. Ionic strength dependence of the equilibrium constant at the field strengths indicated. Experimental points: conditions as in fig. 3 with different amounts of added KCl. (———) Theoretical dependence according to eq. 4 at different field strengths.

4.3. Metmyoglobin, methemoglobin

Figs. 5 and 6 show examples of the optical absorption changes during an electric field pulse in metmyoglobin and methemoglobin solutions. The fast optical absorption change is dependent on the polarization angle of the light relative to the electric field. The positive absorbance changes in both proteins at a polarization angle $\alpha = 0°$, the disappearance of the absorption change at 54.7° ($3\cos^2\alpha - 1 = 0$) and the negative change at 90° are a clear indication for electrodichroism due

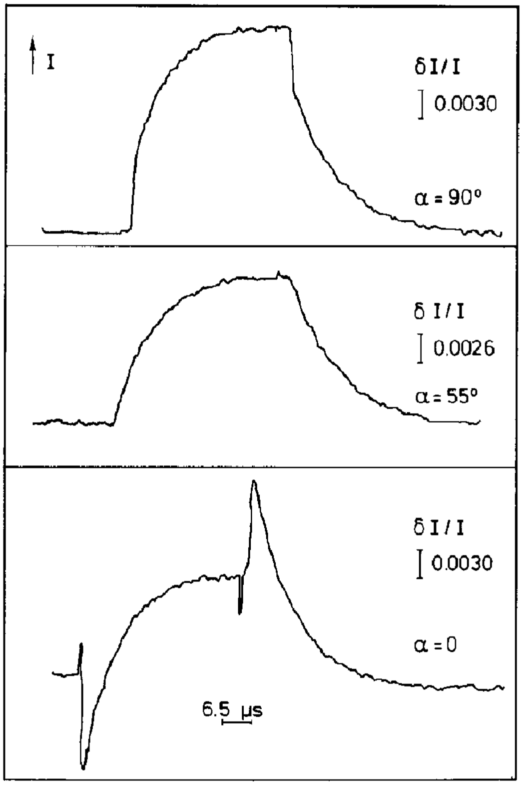


Fig. 5. Dissociation field effect of the acid-alkaline transition in methemoglobin. Change of light intensity vs. time at different polarization angles α of the light relative to the electric field. Conditions: human methemoglobin, 5.3 μM heme, no buffer, pH 8.9, 20 ° C, wavelength 405 nm; electric field strength 53 kV/cm.

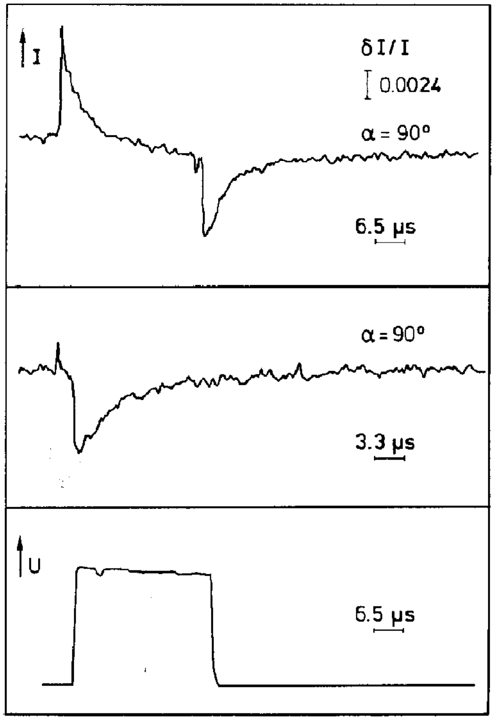


Fig. 6. Dissociation field effect of the acid-alkaline transition in metmyoglobin. (Upper and middle trace) Change of light intensity vs. time at polarization angle $\alpha = 90°$ of light relative to the electric field; (middle trace) relaxation at the end of the field pulse; (lower trace) time course of the electric field pulse. Conditions: sperm whale metmyoglobin, 8 μM heme, no buffer, pH 8.75, 20 ° C, wavelength 405 nm; field strength 50.2 kV/cm.

to the orientation of the protein in the electric field [15].

The slower, time-resolved relaxation process is independent of the polarization angle and can be attributed to the field-induced acid-alkaline transition.

It has been shown previously [10] and is substantiated in the present experiments that:

(i) the reciprocal relaxation times increase linearly with OH^- concentration but are independent of total protein concentration.

(ii) The relaxation amplitudes reach a maximum value at a pH equal to the pK value of the reaction.

These findings are characteristic for a buffered reaction. The buffering, which occurs without added external buffer, must be due to protolytic groups of the protein. An unexpected result is the observation that, in the presence of an electric field, the equilibrium is shifted towards the protonated form in metmyoglobin, but towards the deprotonated form in methemoglobin.

This behavior, however, does not reflect the true electric field dependence of the acid-alkaline transition. Due to the presence of internal buffering groups the observed relaxation amplitudes lead to an apparent change of the equilibrium constant which contains the field effect of these groups. The determination of the true field effect of the acid-alkaline transition is only possible by adding buffers with known field dependence.

The presence of external buffers overcompensates the field effect of the internal buffers. The addition of buffers, however, also increases the ionic strength. Table 1 shows data of the apparent field dependence of the equilibrium constant in different buffers evaluated from the relaxation amplitudes according to

$$\delta \ln K_{\mathrm{app}} = \frac{\delta I}{I} \cdot \frac{1}{2.3 \Delta \varepsilon d} \left(\frac{1}{[\mathrm{FeH_2O}]} + \frac{1}{[\mathrm{FeOH}]} \right) \tag{6}$$

with an optical light path $d = 1.0$ cm (small cell) and the following values for the difference $\Delta\varepsilon$ of the absorption coefficients: metmyoglobin $6.5 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$ (405 nm); methemoglobin 4.5×10^3 (580 nm), $6.1 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$ (405 nm).

The concentrations of $\mathrm{FeH_2O}$ and FeOH are determined from the total protein concentration and the measured pH values, using pK values of 9.0 for metmyoglobin and 8.8 for methemoglobin [19,20].

Assuming equivalent internal buffering groups, we have the following three reactions coupled through the common $\mathrm{OH^-}$ ligand

$$\begin{aligned} &\mathrm{FeH_2O + OH^- = FeOH + H_2O}; \quad K \\ &\mathrm{XH + OH^- = X + H_2O} \quad K_{\mathrm{X}} \text{ internal buffering groups} \\ &\mathrm{ZH + OH^- = Z + H_2O} \quad K_{\mathrm{Z}} \text{ external buffer} \end{aligned} \tag{7}$$

All equilibrium constants refer to the hydrolysis reaction, i.e., $K = [\mathrm{FeH_2O}][\mathrm{OH^-}]/[\mathrm{FeOH}]$, etc.

$\delta \ln K_{\mathrm{app}}$ in eq. 6 can be calculated from the linearized equilibrium relations yielding

$$\delta \ln K_{\mathrm{app}} = \delta \ln K - \delta \ln K_{\mathrm{X}} \frac{\beta}{1 + \beta + \gamma} - \delta \ln K_{\mathrm{Z}} \frac{\gamma}{1 + \beta + \gamma} \tag{8}$$

with

$$\beta = \frac{1/[\mathrm{OH}]}{1/[\mathrm{X}] + 1/[\mathrm{XH}]}; \quad \gamma = \frac{1/[\mathrm{OH}]}{1/[\mathrm{Z}] + 1/[\mathrm{ZH}]}$$

Table 1

Electric field effect of the acid-alkaline transition in human methemoglobin and sperm whale metmyoglobin: apparent change of equilibrium constant, $\delta \ln K_{\mathrm{app}}$, in different buffers

Conditions: [hemoglobin], 5 μM; [myoglobin], 8 μM heme; [buffer], 2 mM; pH 8.7–8.9; field strength 45–48 kV/cm; observation wavelength 405 nm.

buffer	Methemoglobin	Metmyoglobin
No external buffer	−0.041	+0.028
m-Chlorophenol	−0.056	–
Veronal	−0.062	+0.022
Glycine	+0.105	+0.014
Tris	−0.095 ± 0.01	±0.0

At sufficiently high buffer concentrations, i.e. $\gamma \gg \beta \gg 1$, one obtains $\delta \ln K_{\mathrm{app}} = \delta \ln K - \delta \ln K_{\mathrm{Z}}$. The relaxation amplitude becomes independent of $\delta \ln K_{\mathrm{X}}$ of the internal buffering groups and linearly dependent on $\delta \ln K_{\mathrm{Z}}$ of the added buffer.

We have established for the analogous case of

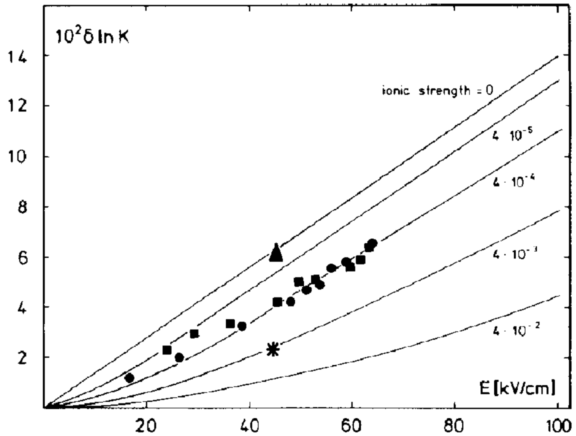


Fig. 7. Dissociation field effect of methemoglobin and metmyoglobin. Field strength dependence of the apparent change of the equilibrium constant. Experimental points: (■, ●) human methemoglobin; absolute values of $\delta \ln K_{\mathrm{app}}$ without external buffer; conditions: concentrations 65 and 83 μM heme, pH 8.8, 20 °C, wavelength 580 nm. (▲) Human methemoglobin; absolute value of $\delta \ln K_{\mathrm{app}}$ in veronal and *m*-chlorophenol buffer. (*) Sperm whale metmyoglobin; $\delta \ln K_{\mathrm{app}}$ in veronal buffer. Conditions as in table 1. (———) Theoretical dependence according to eq. 4 for a reaction of the type $\mathrm{A^+ + B^- = AB}$ at different ionic strengths.

the temperature dependence that the relaxation amplitude in temperature-jump experiments with methemoglobin – metmyoglobin reacts too fast to be investigated with the conventional Joule heating temperature-jump method – is indeed linearly dependent on the reaction enthalpies of the added buffers [21].

Fig. 7 shows the field strength dependence of the absolute value of $\delta \ln K_{app}$ for methemoglobin in unbuffered solution and values for both proteins in *m*-chlorophenol and veronal buffers, which correspond to the true $\delta \ln K$ of the acid-alkaline transition (see section 5). For comparison with theory, the field strength dependence of the equilibrium constant for a reaction $AB = A^- + B^+$ at various ionic strengths according to eq. 4 is added in fig. 7.

5. Discussion

We have presented data on the ionic strength dependence of the dissociation field effect of 2,6-dinitrophenol in aqueous solution. The reaction is of the type $AH = A^- + H^+$ and the increase in ionic dissociation in the presence of a high electric field is monitored through the optical absorption of the chromophore A^-. There is little detailed information as to whether optical absorption and conductivity measurements give the same results: Onsager's criterion for bound and unbound states is the Bjerrum radius, where there is a minimum in the distribution density of counterions, however, this parameter is not likely to be involved in differentiating the protonated and unprotonated chromophore. Nevertheless, all measurements in aqueous solution at very low ionic strength have so far shown that Onsager's theoretical description is consistent with the available data, yielding a linear dependence of the equilibrium constant as a function of electric field strength according to eq. 1.

The linear field strength dependence is only a limiting law at sufficiently high fields, where there is no further screening of the reactive site due to the ionic atmosphere. Our data show that there is a strong influence of the ionic strength on the dissociation field effect. The extent of ionic dissociation in the presence of high fields is reduced at higher concentrations of added neutral salt. In the system investigated, we are able to evaluate the field effect up to ionic strengths of about 0.01 M.

In figs. 3 and 4 the data are compared with the Onsager-Liu theory; agreement is found within the experimental uncertainties. Systematic errors in the determination of $\delta \ln K$ may arise especially from the limited accuracy in pH measurement and in the calibration factors. Comparing figs. 3 and 4 we thus conclude that the extended Onsager theory is fully consistent with the available data.

The data show furthermore that any quantitative estimation of field effects has to take into account the influence of ionic strength. This is especially of importance for biopolymer systems. A decrease of field effects with increasing salt concentration has been observed with polynucleotides [22,23], the results being interpreted under the special aspect of counterion condensation and a theory for these polyelectrolytes has been developed [24].

Eq. 2 is supposed to be valid for symmetrical electrolytes only. We also apply it to the more complex proton transfer reaction in the heme-proteins, in order to obtain a more quantitative estimate of the effective charges involved.

The acid-alkaline transition in the Fe(III) heme-proteins is a hydrolytic reaction, which is influenced by high electric fields. The effect is approximately in the same range as with simple electrolytes. There is a marked difference: while the equilibrium in metmyoglobin is shifted to the acid form in high electric fields, it is shifted to the alkaline form in methemoglobin. This result is unexpected since the reaction site, i.e., the heme group, and the immediately neighboring groups such as the proximal and distal imidazoles are identical in both proteins.

We can exclude the possibility that the field effect of other protolytic groups which are coupled to the acid-alkaline transition via the common reaction partner is responsible for the different sign. The analysis is as follows:

Assuming equivalent internal buffering groups, the observed field effect is given by $\delta \ln K_{app} =$

$\delta \ln K - \delta \ln K_X$, where $\delta \ln K$ corresponds to the acid-alkaline transition and $\delta \ln K_X$ to the field effect of the internal groups (cf. eq. 8 with $\gamma = 0$). Different values of $\delta \ln K_X$ in myoglobin and hemoglobin could thus lead to the observed behavior. In order to determine the true field effect of the transition, we have performed measurements with added buffer. Under such conditions, the effect of the internal buffering groups is overcompensated by the external buffer and the observed field effect is given by $\delta \ln K_{app} = \delta \ln K - \delta \ln K_Z$ where $\delta \ln K_Z$ denotes the field effect of the external buffer (cf. eq. 8 with $\beta \ll \gamma$).

The validity of this relation has been verified in temperature-jump relaxation experiments with methemoglobin, where a linear dependence of the relaxation amplitude and the reaction enthalpy of the buffer is found [21].

For the field-pulse-relaxation experiments we have the following situation (cf. table 1):

'Tris' is a buffer of the type $\text{Tris-H}_2\text{O} = \text{Tris-H}^+ + \text{OH}^-$ which should exhibit a positive field effect ($\delta \ln K_Z > 0$). Veronal and *m*-chlorophenol are buffers with no net charge separation, i.e., $\text{Z}^- + \text{H}_2\text{O} = \text{ZH} + \text{OH}^-$ and no field dependence of the equilibrium constant is expected. This has been tested in field-jump experiments using *m*-nitrophenol, which can be followed spectrophotometrically. Measurements in 1 : 1 water/ethanol solution, where the pK value is 9.4 [17], give no field effect.

The data in table 1 show that $\delta \ln K_{app}$ in Tris buffer is shifted in the same direction, i.e., to more negative values in methemoglobin and less positive values in metmyoglobin in approximate agreement with a value of $\delta \ln K_Z = 0.035$ at 46 kV/cm estimated from the Onsager theory at an ionic strength of 1 mM. The values of $\delta \ln K_{app}$ in veronal and *m*-chlorophenol buffers should be equal to the true field effect of the acid-alkaline transition, since $\delta \ln K_Z = 0$. Under these conditions the field effects still have opposite signs in methemoglobin and metmyoglobin. We therefore conclude that opposite electric charges are effective at the reaction site. There is no correlation with the net charge of the protein molecule, since at pH 8.8 both proteins are negatively charged, the isoelectric points being $\text{pH}_i = 7.5$ for methemoglobin and 8.05 for metmyoglobin [25,26].

A quantitative estimation of the charges involved in the field effect depends on the applicability of the extended Onsager theory to unsymmetrical electrolytes and on an independent knowledge of the dielectric constant.

From fig. 7 is is seen that the field dependence of $\delta \ln K_{app}$ in buffered solution is of the same type as that expected from the Onsager theory. The positive field effect of metmyoglobin, $\delta \ln K = 0.022$ ($E = 46$ kV/cm) in veronal at an ionic strength of 1 mM corresponds quite well to the theoretically expected value for a reaction with singly charged ions in aqueous solutions. We thus assume that it is only the positive charge on the iron in the heme complex which is relevant for the field effect, and that the dielectric constant of water may be used in the evaluation.

In methemoglobin, on the other hand, negative charges of the protein must be involved, in order to account for the negative field effect of $\delta \ln K = -0.059 \pm 0.003$ ($E = 46$ kV/cm) in *m*-chlorophenol and veronal buffers. Using the dielectric constant of water, a number of about four negative charges results. The charged groups are expected to be rather close to the heme iron, since – compared with the Bjerrum radius – distant groups should have no influence as is inferred from preliminary results on the field effect of the azo dye zwitterions. Experiments with water-soluble hemes, which carry additional charges, such as Fe-uroporphyrins, are in progress. Data on such systems would give more precise information on the influence of charges at a defined distance from the reaction site.

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