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The application of pH-sensitive spin labels to studies of surface potential and polarity of phospholipid membranes and proteins

V.V. Khramtsov [1],*, D. Marsh [1], L. Weiner [2] and V.A. Reznikov [3]

[1] *Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, Göttingen (Germany),* [2] *Weizmann Institute, Chemical Physics Department, Rehovot (Israel) and* [3] *Novosibirsk Institute of Organic Chemistry, Novosibirsk (USSR)*

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The effects of pH titration on the EPR spectra of imidazolidine nitroxides located at the surface of mixed bilayers composed of dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylcholine (DMPC), and at the surface of the protein, human serum albumin (HSA), have been investigated. It is found that the shift in pK_a of the amino group of the imidazolidine radical from its value of 4.6 in water depends both on the interfacial polarity (ΔpK_a^{pol}) and on the electrostatic surface potential (ΔpK_a^{el}) when it is positioned at the bilayer/water interface by an anchoring hydrocarbon tail. The polarity shift is determined to be: $\Delta pK_a^{pol} = -1.3$ units at the surface of DMPC bilayers at 17°C, corresponding to an effective interfacial dielectric constant of $\varepsilon \approx 37$, and depends on the temperature with a coefficient of $d\Delta pK_a^{pol}/dT \approx -0.01$ per degree. The electrostatic shift at the surface of DMPG bilayers is $\Delta pK_a^{el} = +1.6$ units in 0.1 M KCl, which corresponds to an electrostatic surface potential of −95 mV. This electrostatic shift depends strongly both on ionic strength and on the fraction of charged lipid in the DMPC/DMPG mixtures, in a manner that agrees with the predictions of electrostatic double-layer theory. It is found that the shift in pK_a of an imidazolidine radical covalently bound at the surface of HSA is determined mainly by the surface electrostatics ($\Delta pK_a^{pol} \approx 0$) and corresponds to an electrostatic potential of +33 mV in 0.01 M KCl at a pH below the isoelectric point of the protein.

Introduction

It has been shown that the EPR spectra of nitroxyl radicals containing ionizable groups are sensitive to the pH of the medium [1–3]. Those spin labels most useful as pH-probes are imidazolidine radicals, which allow one to measure pH in the range from 0 to 14 with an accuracy of 0.05 pH units [3,4]. Recently these radicals have been used for monitoring intraliposomal pH during transmembrane proton transport [5]. It seems to be of considerable interest to use pH-sensitive spin labels for measuring pH at the surface of membranes and proteins because, amongst other reasons, this allows one to estimate the surface polarity and the electrostatic surface potential. In early studies by Fromherz and coworkers (reviewed in Ref. 8), pH-sensitive fluorescent probes have been used to measure surface potentials, and the interfacial ionization equilibria have been analysed in detail by Fernández and Fromherz [28] to yield the shifts in pK_a of such probes that arise from the lower polarity at the interface.

The electrostatic surface potential plays a significant role in many biological functions such as transmembrane transport, membrane–protein interactions, and enzyme catalysis, etc. The methods applied for measuring surface potential either can be indirect, as in the estimation of ζ-potential by studies of electrophoretic mobility or in conductance measurements [6,7], or can be direct approaches using NMR methods [6], fluorescence spectroscopy [6–8,28], or spin probe EPR [9–11]. The advantages of spin probes lie mainly in the possibility of a direct spectral readout related to the location of the probe, and the ability to handle opaque or highly scattering samples on the one hand, and the sensitivity relative to NMR on the other hand. The EPR spectra of nitroxyl radicals of the type previously used in Refs. 9–11 are not intrinsically sensitive to pH (nor to electrostatic potential) in homogeneous solution. Their use for estimation of surface potential, or of surface polarity, depends upon changes in interaction

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* Permanent address: Institute of Chemical Kinetics and Combustion, 630090 Novosibirsk, USSR.

Abbreviations: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; HSA, human serum albumin.

Correspondence: D. Marsh, Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-3400 Göttingen, Germany.

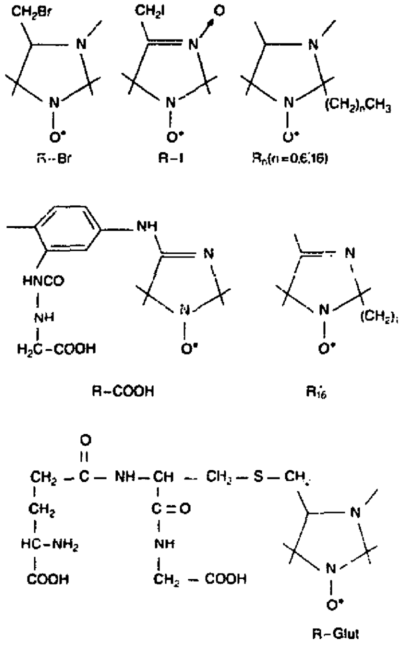
between the radical and membrane phase, either directly with surface potential or indirectly with pH. Such methods have, so far, not proved amenable to measuring electrostatic potentials at the surface of proteins.

The application of spin labels with intrinsic spectral sensitivity to pH seems promising for the study of electrostatic surface potential of membranes and particularly of proteins. In the present paper we have used pH-sensitive imidazolidine radicals for measuring the electrostatic potential and the local polarity at the surface of negatively charged phospholipid model membranes and at the surface of a protein – human serum albumin (HSA).

Materials and Methods

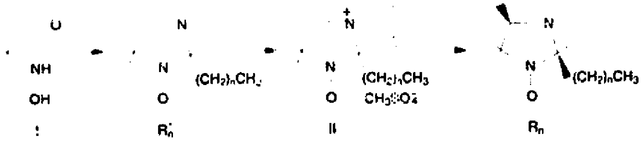
Spin label synthesis. The radicals given in Scheme I were synthesized using published procedures. The synthesis of radical R-Br is described in Ref. 12, that of radicals R_0 and R-I in Ref. 13, of radical R-Glut in Ref. 5 and of radical R-COOH in Ref. 14. The reaction scheme for the synthesis of radicals R_n and R_n^* is given in Scheme II. The radical R_6^* was synthesized as described in Ref. 25 by condensation of hydroxylaminoketone, I, with methylheptylketone in the presence of ammonium acetate, followed by oxidation with MnO_2. The compound, II ($n = 6$), was obtained by reaction of the radical R_6^* with dimethylsulphate [25]. The compounds R_{16}^* and II ($n = 16$) were synthesized similarly, as described in Ref. 25. The radicals R_6 and R_{16} were synthesized as described in Ref. 26 by reduction of the compounds II ($n = 6$ and $n = 16$, respectively) with $NaBH_4$ in water-ether solution with a yield of 90%. During the reduction a mixture of diastereoisomers is obtained, which was separated by chromatography on a silica gel column and eluted with a mixture of ethyl acetate and hexane (1:6, v/v). The diastereomer shown in the scheme was used in the present work. Elemental analyses found (calculated) were C 70.1(70.3), H 11.3(11.3), N 11.6(11.7); C 76.1(76.0), H 12.5(12.4), N 7.4(7.4); C 70.5(70.7), H 12.4(12.2), N 10.9(11.0); and C 76.1(75.9), H 13.0(12.9), N 6.9(7.1) for the compounds R_6^*, R_{16}^*, R_6 and R_{16}, respectively.

Modification of HSA by R-COOH radical. The procedure for covalent linkage of R-COOH to human serum albumin follows Ref. 15 and was performed as follows. The carboxyl group of the radical was activated



Scheme I.

by using dipentofluorophenylcarbonate (DFPC) which was synthesized at the Institute of Proteins of the USSR Acad. Sci. For this purpose, 25 mg (0.063 mmol) of DFPC and 7 μl (0.05 mmol) of triethylamine were added to a solution of 16 mg (0.05 mmol) of R-COOH in 200 μl of dimethylformamide (DMF) and the mixture was incubated for 30 min at 20°C. The analysis of the reaction mixture was performed by thin-layer chromatography on silica gel (Merck, N 5554): the R_f is 0.05 for the initial radical and 0.17 for the activated one (in heptane/acetone (2:1, v/v)). The reaction mixture was diluted with 1 ml of diethyl ether and 5 ml heptane, upon which the activated radical precipitated as an oil, and was washed with hexane and dried in vacuo. The activated radical was bound to HSA as follows: A solution of 16 mg of activated radical in 120 μl of DMF was added dropwise to a solution of 60 mg of HSA (Reanal, Budapest, Hungary) in 1.2 ml of 2% $NaHCO_3$. The reaction mixture was incubated for 24 h at 20°C. After the incubation, unreacted radical was



Scheme II.

removed by gel filtration (Sephadex G-50, Pharmacia, Uppsala, Sweden). The degree of modification was 0.5 ± 0.1 nitroxyl groups per protein.

Modification of HSA by R-Br and R-I radicals. A solution of 2 ml of the radical ($c = 0.075$ M) and HSA (5 mM) in 0.01 M NaOAc buffer (pH 5.0), was incubated for 16 h with stirring at 2°C. After incubation, the unreacted radical was removed by gel filtration (Sephadex G-25, Pharmacia, Uppsala, Sweden). The protein concentration was determined by the Lowry method [16] and the radical concentration was determined from the double integral of the EPR spectrum. The degree of modification was 1.1 ± 0.2 nitroxyl groups per protein.

Preparation of phospholipid multilayers. Solutions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Fluka, Buchs, Switzerland), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) (Avanti, Birmingham, Alabama) and radical R_{16} in CH_2Cl_2 were mixed in the required concentrations, then the total solution was dried under vacuum for 12 h. The dry lipid film was dispersed in the appropriate buffer, introduced into a 100 μl glass capillary and centrifuged at $10000 \times g$ for 5 min. The sedimented multilayers were used for EPR studies.

EPR spectroscopy. EPR spectra were recorded on Varian Associates E-line and on Bruker ER 200D-SRC 9 GHz EPR spectrometers. The lipid samples were thermostatted by nitrogen gas flow, using a double-wall quartz sample dewar. Temperature was measured using a fine-wire thermocouple positioned at the top of the cavity and dipping into silicone oil contained in a standard 4-mm quartz EPR tube in which the sample capillary was located. Subtractions were performed on digitized spectra, essentially as described in Ref. 23.

Results and Discussion

pK_a of the radical R_{16} in DMPC bilayers

The intrinsic pK_a^0 of the nitrogen atom N-3 in the heterocycle of the imidazolidine radical R_{16} is expected to have a value of approximately 4.5 in water. This value cannot be measured directly due to the very low solubility of this radical in water and was estimated from the pK_a values for radicals R_0 (p$K_a^0 = 4.7$) and R_6 (p$K_a^0 = 4.6$). The addition of a number of methylene group substituents at position 2 of the heterocycle cannot change significantly the value of the pK_a of the radical because of the relatively large distance of these groups from the N-3 atom.

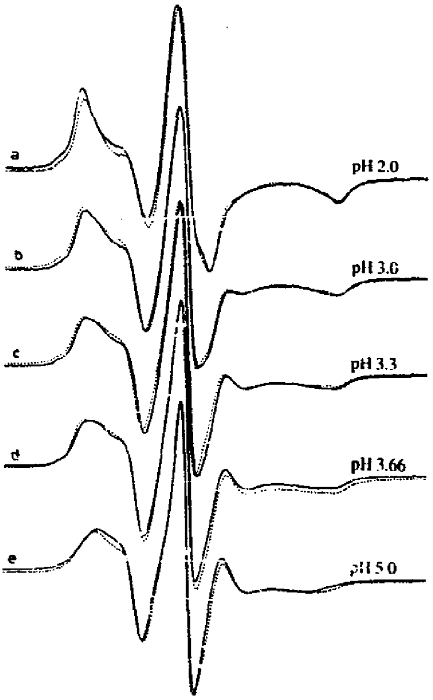


Fig. 1. EPR spectra of the radical R_{16} as a function of pH in DMPC bilayer dispersions (10 mM NaOAc buffer) at 17°C. Full lines are the original experimental spectra and the dashed lines are the results of spectral subtraction and addition. Dashed lines: (a) spectrum at pH 3.3 minus 48% of the spectrum of the unprotonated radical at pH 5.0; (b) 68% of the spectrum of the protonated radical at pH 2.0 plus 32% of the spectrum of the unprotonated radical at pH 5.0; (c) 52% of the spectrum of the radical at pH 2.0 plus 48% of the spectrum of the radical at pH 5.0; (d) 35% of the spectrum of the radical at pH 2.0 plus 65% of the spectrum of the radical at pH 5.0; (e) spectrum at pH 3.3 minus 52% of the spectrum of the protonated radical at pH 2.0. The spectra were normalized to the same double integrated intensity for the spectral subtractions and additions, but are scaled to the same maximum lineheights in the plots. Total scan width = 10 mT.

Fig. 1 shows the effect of pH on the EPR spectra of the radical R_{16} incorporated in DMPC multilayers. There are spectral changes in the pH range from 2 to 4.5 due to protonation of the N-3 atom of the R_{16} radical. For the R_{16}^* radical incorporated in DMPC multibilayers, on the other hand, we did not observe any spectral changes over the same pH range, in agreement with the very acidic pK_a (about 1) of the N-3 atom of this particular imidazolidine radical. It should be noted that the main reason for the pH sensitivity of the EPR spectrum of radical R_{16} in liposomes is not changes of the intrinsic hyperfine parameters of the N-O fragment on protonation of the N-3 atom, as is the case in homogeneous solution, but is the change in interaction between the radical and membrane on protonation of the imidazolidine moiety. The exact molecular nature of this change is not known with certainty, but it might result from a change in the vertical location of the probe (as in spin-labelled fatty acids [29]) or from a direct interaction of the charged moiety with the phospholipid headgroups. In any case, it is clear that it is the protonated, i.e. positively charged, form of the probe which has the greater restriction of its rotational mobility.

An interfacial pK_a^i of 3.3 ± 0.2 was estimated for R_{16} in DMPC at 17°C by fitting the EPR spectra of the radical at intermediate pH values as a linear combination of normalized (second integral fixed to unity) spectra obtained at the titration extremes of pH 2 and pH 5. The spectrum that gives equal fractions of the two components in the total spectrum corresponds to $pH = pK_a^i$ (see Fig. 1). Fitting the fraction, f, of the high pH component to a conventional titration curve:

$$f = 1/(1 + [H^+]/K_a^i) \tag{1}$$

provided a check on the estimate of the interfacial pK_a. These results are given in Fig. 2 and yield a value of $pK_a^i = -\log_{10} K_a^i = 3.3$. A simpler, empirical analysis from the pH-dependence of the ratio of peak intensities of the central ($m_I = 0$) and low-field ($m_I = +1$) EPR components was also found to yield reliable values of the pK_a^i, as illustrated in Fig. 3 which is given later below. This method gave a value of $pK_a^i = 3.25$ for the radical R_{16} in DMPC at 17°C, in agreement with the more direct analysis. Because of its simplicity and reliability the lineheight ratio method was used in the subsequent analysis.

The interfacial pK_a^i of the radical at the polar/apolar interface of the bilayer is shifted from the intrinsic pK_a^0 by an amount ΔpK_a^i which depends in the general case both on the interfacial polarity and on the surface electrostatics [8,18,28]:

$$pK_a^i = pK_a^0 + \Delta pK_a^{el} + \Delta pK_a^{pol} \tag{2}$$

The electrostatic shift, ΔpK_a^{el}, in pK_a is related to the surface potential, ϕ, by the following equation [18]:

$$\Delta pK_a^{el} = -e\phi/\ln(10)kT \tag{3}$$

where T is the absolute temperature, e is the elementary charge and k is Boltzmann's constant. In the case of uncharged DMPC bilayers this electrostatic shift is equal to zero.

The polarity-induced shift is determined by destabilization of the forward association reaction $R_{16} + H^+ \rightleftharpoons R_{16}H^+$ by the lower polarity at the interface [8,28], and for reactions involving the dissociation of a molecular acid (e.g. stearic acid in DMPC bilayers [10,11]) it has the opposite sign. Titration of the radical R_6 in water-ethanol solutions results in a linear dependence of ΔpK_a^{pol} on the dielectric constant, ε, over the range of ε from 30 to 78, namely:

$$\Delta pK_a^{pol} = 0.032(\varepsilon - \varepsilon_{H_2O}) \tag{4}$$

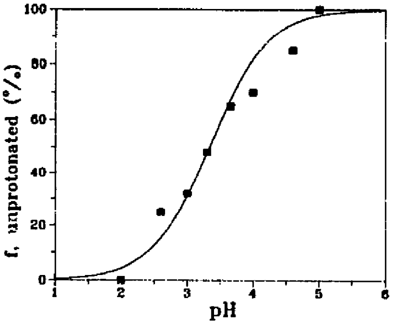


Fig. 2. pH dependence of the fraction, f, of the unprotonated form of the radical R_{16} in DMPC bilayer dispersions at 17°C. The values of f were determined from the EPR spectra as indicated in Fig. 1. The solid line is a non-linear least-squares fit of the data to Eqn. 1, yielding a value of $pK_a^i = 3.3$.

Hence the measurement of the negative shift in pK_a ($\Delta pK_a^{pol} = -1.3$) of radical R_{16} in DMPC bilayers at 17°C allows an estimate of the effective dielectric constant at the membrane surface in the region of the radical heterocycle. Using eqn. 4 this gives a value of $\varepsilon^i = 37$, corresponding to a lower polarity at the membrane interface than in bulk solution (cf. Refs. 8 and 28). This polarity-induced shift is temperature dependent as seen in Fig. 3 (given below) and corresponds to a temperature coefficient of $d\Delta pK_a^{pol}/dT \approx -0.01$ per degree. For a carboxylic acid spin probe in DMPC bilayers a similar temperature-induced shift in pK_a^i was found [11] but of the opposite sign to that found here, as is expected for the dissociation of a molecular acid as opposed to a cationic acid.

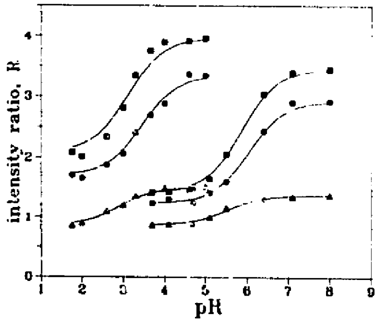


Fig. 3. pH dependence of the ratio of the EPR spectral peak height intensities of the central ($m_I = 0$) and low-field ($m_I = +1$) components for the radical R_{16} in DMPC bilayer dispersions (three left-hand curves) and in DMPG bilayer dispersions (three right-hand curves) in 10 mM NaOAc buffer. ●, Data at 9°C; ■, at 17°C; ▲, at 50°C. The solid lines represent non-linear least-squares fits of the data to Eqn. 5, where α is determined from the spectra at the extremes of the titration to have values of: 0.66, 0.83 and 1.39 at 9, 17 and 50°C, respectively.

Measurement of the surface potential of bilayers containing DMPG

The pH titration of the radical R_{16} in DMPG bilayers is compared with that in DMPC bilayers in Fig. 3. The titration is expressed in terms of the empirical lineheight ratio $R = h(0)/h(+1)$, where $h(0)$ and $h(+1)$ are the lineheights of the central and low-field components, respectively. The titration curves have been fitted to the standard expression (cf. eq. 1) [17]:

$$R = \left(R_{\max} + R_{\min}\alpha[\mathrm{H}^+]/K_a^i\right)/\left(1 + \alpha[\mathrm{H}^+]/K_a^i\right) \quad (5)$$

where $R_{\max}$ and $R_{\min}$ are the values of R for the fully deprotonated and the fully protonated species, respectively, and $\alpha = h_{\min}(+1)/h_{\max}(+1)$ is the ratio of the normalized low-field lineheights of the protonated and deprotonated species, respectively.

When the radical R_{16} is incorporated in negatively charged DMPG bilayers a positive shift is observed in the $\mathrm{p}K_a^i$ of the radical, namely $\Delta \mathrm{p}K_a^i = +1.3$ at 17°C, which is due both to the polarity-induced shift and to the electrostatic shift. Using the polarity-induced shift measured above in DMPC yields a value for the electrostatically induced shift of $\Delta \mathrm{p}K_a^{el} = +2.6$. This electrostatic shift then allows estimation of the surface potential, ϕ, by using Eqn. 3; a value of $\phi = -150$ mV is obtained for DMPG in 10 mM NaOAc at 17°C.

The ionic strength dependence of the surface potential of DMPG bilayers measured in the above manner is given in Fig. 4. At 0.1 M KCl the surface potential is found to be -95 mV. This is in agreement with a previous determination using a different spin-label method which gave a value of $\phi \approx -100$ mV for DMPG bilayers in the gel phase at a similar ionic strength [20].

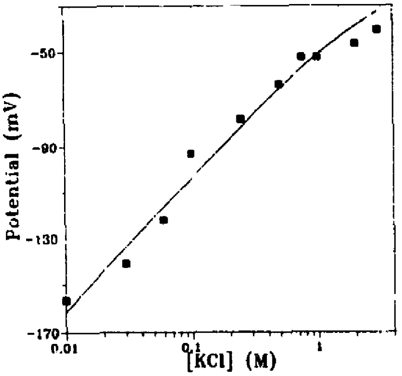


Fig. 4. Dependence of the electrostatic surface potential, ϕ, of DMPG bilayer dispersions in 10 mM NaOAc buffer on KCl concentration. Experimental points (■) were determined from the electrostatic shift in $\mathrm{p}K_a^i$ of the radical R_{16} as illustrated in Fig. 3 and the solid curve is a non-linear least-squares fit to Eqn. 6, with an area per lipid molecule of $A_0 = 1.17$ nm^2.

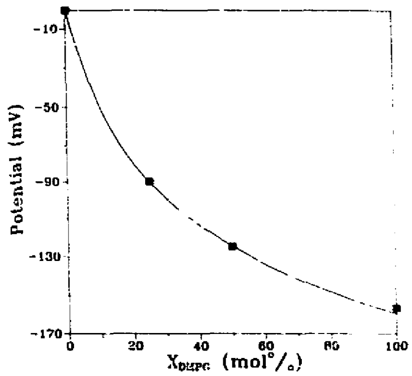


Fig. 5. Dependence of the electrostatic surface potential, ϕ, of DMPG-DMPC mixed lipid dispersions in 10 mM NaOAc buffer on the mole fraction of charged lipid (DMPG) in the bilayer. Experimental points (■) were determined from the electrostatic shift in $\mathrm{p}K_a^i$ of the radical R_{16} as illustrated in Fig. 3 and the solid curve is a non-linear least-squares fit to Eqn. 6, with an area per lipid molecule of $A_0 = 1.20$ nm^2.

The data in Fig. 4 have been fitted to the standard Gouy-Chapman equation from electrostatic double-layer theory [18]:

$$\phi = (2kT/e)\sinh^{-1}\left\{\sigma/\left(\sqrt{[8000\epsilon_b\epsilon_0 N_A kTI]}\right)\right\} \quad (6)$$

where σ is the surface charge density, ϵ_b and ϵ_0 are the dielectric constant in the electrical double-layer and the permittivity of free space, respectively, and I is the ionic strength. The surface charge density is given by: $\sigma = -eX/A_0$, where X is the mole fraction of negatively charged lipid (DMPG) and A_0 is the area per lipid molecule. The fit requires an artificially high value for the area per lipid molecule ($A_0 = 1.17$ nm^2, instead of 0.48 nm^2 as determined for DMPG in the gel phase by X-ray diffraction studies [19]). This over-estimation of the electrostatic surface potential for highly charged membranes by Gouy-Chapman theory is well-known [20] and is attributable at least in part to the simplifying assumptions made in this approach (see Ref. 18 for a discussion).

The electrostatic surface potential was also measured as a function of surface charge density in bilayers composed of mixtures of the negatively charged lipid DMPG with the zwitterionic lipid DMPC. These results are given as a function of the mole fraction, X, of DMPG in Fig. 5. The decrease in surface potential with decreasing surface charge density is clearly apparent in the figure. These data have also been fitted to the predictions of electrostatic double-layer theory by using Eqn. 6. Again a larger value of $A_0 = 1.20$ nm^2 is required for the area per lipid molecule in order to fit the data with standard Gouy-Chapman theory.

Thus the radical R_{16} extends the class of spin labels with ionizable head groups that are useful for surface electrostatic measurements by EPR [10,11,21] and also extends the pH range of their applicability. It should be noted that the acidity of N-3 in the imidazolidine ring is strongly dependent on the substituent at the C-4 atom [4], and therefore other analogues are available in principle with higher values of the intrinsic pK_a^0 than that of R_{16}. The length of the alkyl chain can be adjusted to improve partitioning, but in dilute membrane suspensions a contaminating free aqueous spin label signal may cause complications unless double-chain probes are used. The ability to work with opaque or highly scattering samples is an advantage. However, it is also something of a necessity for reasons of signal-to-noise ratio. The smaller size of the reporter group may be of advantage in comparison with fluorescent probes, although EPR cannot compete with fluorescence in terms of sensitivity if concentrations are limiting.

Measurement of surface potential of the protein HSA

The electrostatic potential at the surface of a protein can be measured using pH-sensitive radicals by comparing the pK_a of the radical in bulk solution with that at the surface of the protein. In this case, the intrinsic sensitivity of the EPR spectra of the imidazolidine radicals to pH (see Refs. 3 and 4) is exploited. The choice of the length of the linkage between the protonatable group and the point of covalent attachment, and of the intrinsic pK_a^0 of the radical, is important for such determinations. For instance, when the radical R-COOH was covalently bound to HSA (see Materials and Methods) a highly mobile EPR spectrum of the radical was observed (see Fig. 6A). In addition, the pK_a of the bound radical determined by titration of the isotropic hyperfine splitting constant, a_N, (see Fig. 6B) was found to be equal to that of the radical R-COOH in bulk solution ($pK_a = 4.7$). The motional freedom of the bound label suggests that the protonatable imidazolidine moiety is located at a position remote from the protein surface. Therefore, the pK_a of the label is insensitive to the protein electrostatics and remains unshifted from its intrinsic value which is close to the isoelectric point of the protein ($pI = 4.9$ [27]).

When the radical R–Br with a short distance between radical heterocycle and alkylating $-CH_2Br$ group was covalently bound to the SH-group of HSA (see Materials and Methods) a moderately immobilized EPR spectrum of the radical was observed (see Fig. 6A). A clear shift in pK_a of the label covalently bound at the surface of HSA was found relative to that for the label bound to the low-molecular weight compound glutathione in aqueous solution (see Fig. 6B). In order to exclude the possibility that the spectral changes observed might be associated with conformational changes of the protein on changing the pH, the nonprotonatable radical R–I was used as a control. The EPR spectra of R–I in aqueous solution and bound to HSA were insensitive to pH over the range from 1.5 to 6.6.

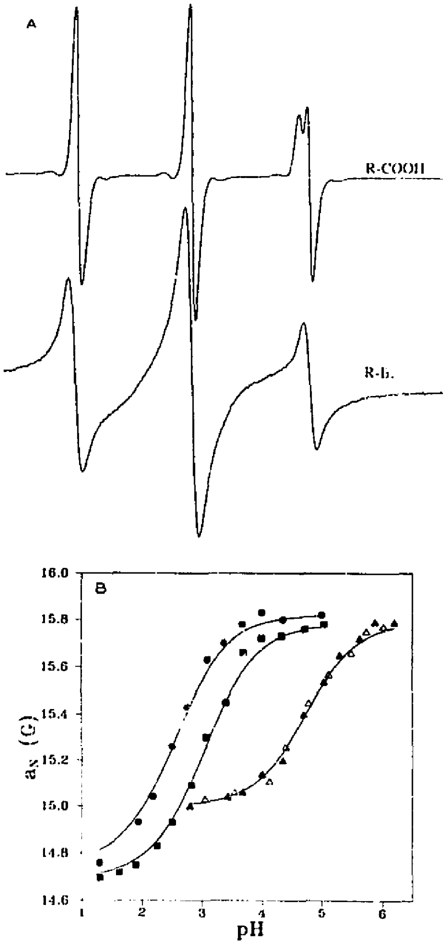


Fig. 6. (A) EPR spectra recorded at 23°C of the radicals R-COOH and R–Br covalently bound to human serum albumin in 0.01 M KCl at pH 4.9 and pH 5.0, respectively. The isotropic hyperfine splitting constant, a_N, is measured as the distance between the baseline crossing points of the low-field ($m_I = +1$) and central ($m_I = 0$) lines. Total scan width = 6 mT. (B) pH dependence of the isotropic hyperfine interaction constant, a_N, for the radicals R-COOH (▲) and R–Br (●) covalently bound to HSA and of the radical R–Br bound to glutathione (■) and of the radical R-COOH alone (△) in 0.01 M KCl at 23°C.

The observed downward shift in pK_a of the R–Br radical could be connected either with the electrostatic potential, or with a lower polarity, at the surface of HSA. To estimate the surface polarity at the position

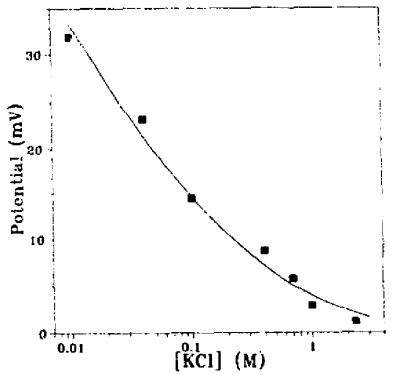


Fig. 7. Dependence of the electrostatic potential at the location of the N-3 atom of the radical R–Br covalently bound to HSA on KCl concentration. Experimental points (■) were determined from the shift in pK_a of the radical as illustrated in Fig. 6 and the solid curve is a non-linear least-squares fit to Eqn. 7, with $r_p = 2.8$ nm, yielding $\Delta r = 0.2$ nm and $\phi_0 = +65$ mV.

of the radical moiety it is possible to use the well-known dependence of the hyperfine interaction constant, a_N, of nitroxyl radicals on polarity [22]. There is found to be very little difference in a_N for the radical R–Br bound to glutathione and to HSA, both in the protonated and in the unprotonated forms of the radical (see Fig. 6B), indicating that the polarity at the location of the radical bound to HSA is similar to that in bulk solution. Therefore, the main reason for the shift in pK_a of the radical at its binding site on HSA results from the non-zero electrostatic potential at the surface of the protein. The surface potential can be calculated using Eqn. 3, where ΔpK_a^{el} is given by the difference between the pK_a for the radical R–Br bound to HSA and to glutathione. A value of +33 mV is obtained for HSA at pH 2.5–3 in 0.01 M KCl, the sign of which is consistent with the isoelectric point of the protein: p$I = 4.9$.

The dependence on salt concentration of the potential measured at the location of the N-3 atom of the radical bound to HSA is given in Fig. 7. The screening of the potential with increasing ionic strength, I, has been fitted to the linearized Poisson-Boltzmann equation for an isolated spherical surface of radius r_p (see Ref. 18):

$$\phi = \phi_0[\lambda/(\lambda + r_p)] \exp(-\Delta r/\lambda) \quad (7)$$

where $\Delta r = r - r_p$ is the distance of the N-3 atom from the protein surface, ϕ_0 is the surface potential at zero ionic strength ($I = 0$, $\Delta r = 0$), and $\lambda = \sqrt{[\epsilon_b \epsilon_0 kT/(2000 N_A e^2 I)]}$ is the Debye screening length ($\lambda = 0.304/\sqrt{I}$ nm at 294 K for monovalent salt). The data fit well with the N-3 atom of the radical located at a distance $\Delta r = 0.2$ nm from the protein surface, yielding a value of $\phi_0 = 65$ mV for the surface potential in the absence of salt, where a value of $r_p = [3M\bar{v}_p/4\pi N_A]^{1/3} = 2.8$ nm is taken for the radius of the protein [24]. The agreement of the ionic strength dependence with Debye-Hückel theory and the nearly complete screening at a KCl concentration of approx. 2 M further confirm that the shifts in pK_a observed for the radical R–Br bound to HSA are dominated by electrostatic effects and hence give a reliable method of estimating the surface potential.

Conclusions

(1) The hexadecyl-linked imidazolidine radical, R_{16}, has an intrinsic pK_a of approx. 4.6. It can be used to determine both the surface polarity and the surface potential of lipid bilayer membranes. The sensitivity arises from the different interactions of the protonated and deprotonated forms with the membrane environment. It is complementary to the long-chain fatty acid spin labels used previously in that the protonated form is charged and therefore the polarity-induced shifts in pK_a are of the opposite sign.

(2) The activated imidazolidine radical, R–Br, can be covalently linked to nucleophilic side chains on proteins. The intrinsic sensitivity of the imidazolidine radicals to pH can then be used to determine the electrostatic potential at the surface of the protein.

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