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A simple spectroscopic method for studying erythrocyte ghost resealing

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A novel spectroscopic method is described for following the kinetics of resealing of hemolysed erythrocyte ghosts. The procedure is based on the broadening of the EPR spectrum of nitroxyl radicals by paramagnetic ions. The method is used to study the effect of Ca^{2+}, Mg^{2+} and dimethonium ion on the kinetics of resealing.

Introduction

The resealing of hemolysed erythrocyte ghosts is a phenomenon that has been the subject of a number of studies [1]. The standard method of following the degree of resealing is to use a membrane-impermeable marker which is trapped by the closing of the ghost. The subsequent steps in the estimation of trapped marker involve the separation of the membrane system from the media and the determination of the amount of marker, usually by a spectroscopic method. The procedure is time-consuming and the separation and washing of the membranes can result in significant errors. In the present paper a simple spectroscopic method is described for following resealing without the need for separation procedures. The method is based on the broadening of the EPR spectrum of nitroxyl radicals by paramagnetic ions. The kinetics of resealing have been shown to be affected by divalent metal cations [2]. Evidence is presented here to suggest that this effect is predominantly electrostatic in origin.

Methods, Materials and Results

All salts were analytical reagent grade. Erythrocyte ghosts were prepared from freshly donated blood. Erythrocytes were separated by washing three times in 150 mM NaCl/10 mM sodium phosphate buffer at pH 7.4 (PBS) using a clinical centrifuge for 3 min at 3000 rpm. Ghosts were prepared by a slight modification of the method described by Dodge et al. [3]. 1 ml of the washed erythrocytes at 4°C was lyzed by the addition of 40 ml of a solution of 5 mM Tris buffer (pH 8)/0.05 mM EDTA/0.12 mM PMSF. The lyzed sample was centrifuged at 15000 rpm for 10 min in a Sorval RC-5 centrifuge. The membranes were washed twice in this manner and then given a third wash in a solution of the same composition excepting the absence of EDTA. The resulting sample, containing 2–4 mg/ml of protein, was kept at 4°C. When these membranes are incubated at 37°C in 150 mM NaCl/5 mM Tris-HCl buffer at pH 7.4, they reseal [4]. Resealing was followed by the use of oxo-TEMPO, a nitroxide free radical which displays an EPR spectrum having three narrow lines with a hyperfine splitting of −16 gauss in the experimental solutions. The addition of $K_3Fe(CN)_6$ to solutions of oxo-TEMPO broadens the EPR spectrum to an extent dependent on the concentration of ferricyanide (Fig. 1). If resealing occurs in a solution of lyzed ghosts containing oxo-TEMPO the sealed ghosts will contain the spin probe in their internal volume. If $K_3Fe(CN)_6$, which permeates membranes slowly, is added to the solution the resultant EPR spectrum is a superposition of broad lines, due to spin-probe in the bulk solution, and sharp lines, due to spin-probe trapped within resealed ghosts and inaccessible to ferricyanide, Fig. 2. Thus the amplitude of the sharp spectrum is linearly proportional to the total volume contained within resealed ghosts, and its height is

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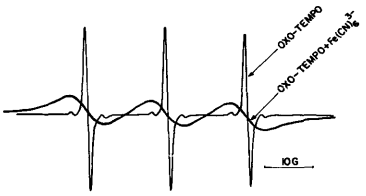

Fig. 1. EPR spectrum of oxo-TEMPO in solution in the absence and presence of the broadening agent $K_3Fe(CN)_6$.

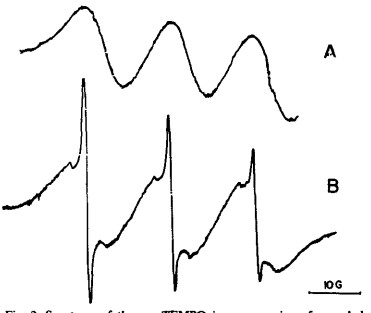

Fig. 2. Spectrum of the oxo-TEMPO in a suspension of unsealed ghosts (A) and partially sealed ghosts (B) in the presence of $K_3Fe(CN)_6$.

readily derived from the observed spectrum (see Fig. 3). In a typical experiment a sample of the stock solution of ghosts was diluted to give a solution containing 150 mM NaCl/5 mM Tris-HCl. The resulting solution was incubated at 37 °C in order to initiate resealing of the ghosts and aliquots taken over a period of about one hour. $K_3Fe(CN)_6$ and oxo-TEMPO were added to each aliquot to give a final concentrations of 90 mM and 10^{-3} M, respectively, and the EPR spectrum recorded (Fig. 4). As shown in Fig. 5, where data from a typical experiment are plotted, resealing shows first-order kinetics, as previously shown by Johnson [4]. The first-order rate constant for resealing at 37 °C under the experimental conditions given above was found to be 0.116 min^{-1} compared to an interpolated value of 0.122 min^{-1} obtained from the results given by Johnson. Thus the present method gives effectively the same kinetic results as the more involved alternative methods. The addition of 270 mM K^+ could conceiv-

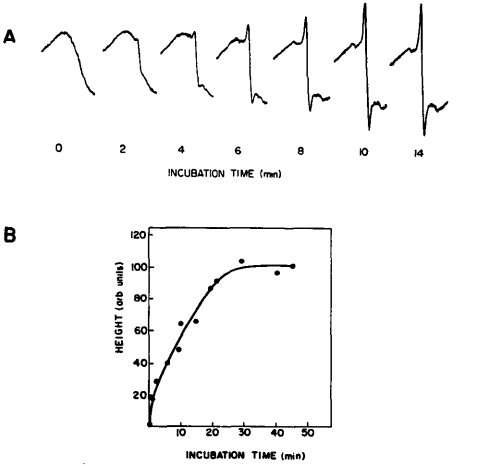

Fig. 3. Observation of ghost resealing: (A) Change of the low-field line of the EPR spectra with time. (B) Graph of the signal height in arbitrary units.

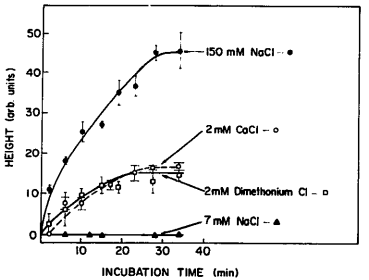

Fig. 4. EPR signal height for solutions of resealing ghosts in various solutions as a function of time.

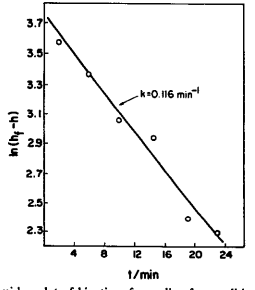

Fig. 5. Semi-log plot of kinetics of resealing for conditions given in text.

ably induce resealing but this does not affect the observed 'internal' signal since any resealing occurring after the addition of $K_3Fe(CN)_6$ will trap oxo-TEMPO and ferricyanide in the vesicle and the internal oxo-TEMPO will contribute only to the broadened EPR line. The method described has a major advantage over previous methods in that no separation steps are required. The aliquot can be as small as 20 μl, the minimum volume needed to give an EPR signal with acceptable signal to noise ratio. Since $Fe(CN)_6^{3-}$ slowly penetrates the sealed ghosts the inner signal undergoes slow broadening with time. Penetration might be via a small persistent hole of a few Ångstroms diameter [5]. There are two simple ways round this difficulty. The time dependence of signal height can be determined as a function of time and extrapolated to zero time. Alternatively the EPR spectrum can be recorded within a very short time after addition of ferricyanide. There is in fact no difficulty in recording spectra within one minute of taking a sample of ghosts, in which time the change in signal height is not more than 3 to 4%.

The method was used to follow the kinetics of resealing in the presence of selected ions. It is known [2] that Ca^{2+} ion accelerates the kinetics of ghost resealing with respect to the observed rate in 7 mM NaCl. In Fig. 4 the rates of resealing in the presence of 2 mM $CaCl_2$ and 2 mM dimethonium chloride are compared using our method. As seen both ions accelerated the rate of ghosts resealing. This effect of dimethonium ions could be anticipated since it has been previously shown that the effect of dimethonium resembles the effect of Ca^{2+} on the separation of the cytoskeleton proteins from the membrane of erythrocytes and it is probably largely electrostatic in origin [6]. Dimethonium ion has been shown not to bind to phospholipid bilayers and its outer layer of methyl groups makes it very unlikely that it binds significantly to any charged groups.

Divalent ions are known to have a far greater shielding effect that monovalent ions on the surface potential produced by surface charge [7]. The repulsive pressure between two surfaces with fixed charge is a function of their surface potentials, ϕ_0. As ϕ_0 drops so does the pressure. The fact that Ca^{2+} and Mg^{2+} affect membrane electrical properties can be partially attributed to shielding effects but specific binding to the membrane cannot be ruled out. Dimethonium does not bind to membranes and the fact that Ca^{2+} and DMT^{2+} have effectively the same accelerating effect on resealing very strongly suggests that both ions act by reducing the surface potential of the membrane.

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