Hemoglobin Affinity for 2,3-Bisphosphoglycerate in Solutions and Intact Erythrocytes: Studies Using Pulsed-Field Gradient Nuclear Magnetic Resonance and Monte Carlo Simulations

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ABSTRACT The diffusion coefficient (D) of 2,3-bisphosphoglycerate (DPG) was measured using pulsed-field gradient (PFG)-^{31}P nuclear magnetic resonance spectroscopy in solutions containing 2.7–5.0 mM hemoglobin (Hb) and a range of DPG concentrations. The dependence of the measured values of D on the fraction of the total DPG in the sample that is bound to Hb enabled the estimation of the dissociation constants (K_d) of complexes of DPG with carbonmonoxygenated, oxygenated, and deoxygenated Hb; the values of K_d (mM), measured at 25°C, pH 6.9 and in 100 mM bis Tris/50 mM KCl, were 1.98 ± 0.26, 1.8 ± 0.5 and 0.39 ± 0.26, respectively. In intact erythrocytes the apparent diffusion coefficient, D_{app}, of DPG was larger in oxygenated and carbonmonoxygenated cells (6.17 ± 0.20 × 10^{-11} m^2s^{-1}) than in deoxygenated cells (4.10 ± 0.23 × 10^{-11} m^2s^{-1}). Changes in intracellular DPG concentration (5–55 mM) in erythrocytes, brought about by incubation in a medium containing inosine and pyruvate, did not result in significant changes in the value of D_{app}; this result supports the hypothesis that DPG binds to other sites in the erythrocyte. Monte Carlo simulations of diffusion in biconcave discs were used to test the adequacy of the values of K_d estimated in solution to describe the binding of DPG to Hb in oxygenated and deoxygenated erythrocytes. The results of the simulations implied that the value of K_d estimated for deoxygenated Hb-DPG was greater than expected from the experiments involving intact erythrocytes. This difference is surmised to be at least partly due to the difficulty of measuring D at low-ligand concentrations. Notwithstanding this shortcoming, the PFG method appears to be suitable for probing interactions between macromolecules and ligands when the K_d is in the millimolar range. It is one of the few techniques available in which these interactions can be studied in intact cells. In addition, the Monte Carlo simulations of the diffusion experiments highlighted important differences between theory and experiment relating to the nature of molecular motion inside the cells.

INTRODUCTION

The most abundant phosphorylated metabolite in many mammalian erythrocytes is 2,3-bisphosphoglycerate (DPG), which in humans occurs in approximately equimolar concentration (5 mM) with hemoglobin (Hb; Benesch and Benesch, 1969; Arnone, 1972). The allosteric binding of DPG to Hb results in a dramatic decrease in the oxygen affinity of the Hb molecule and thus facilitates the delivery of oxygen to the tissues by erythrocytes (Benesch and Benesch, 1969). In humans this effect results from the ~100 times higher binding affinity of the deoxy form (deoxyHb) over the oxy form of the Hb molecule (HbO_2) for DPG (Benesch and Benesch, 1969; Hamasaki and Rose, 1974); one molecule of DPG is bound per tetramer of Hb in both deoxyHb and HbO_2 (Hamasaki and Rose, 1974). Carbon monoxide (CO) can replace O_2 as the ligand in Hb forming carbonmonoxy Hb (HbCO), in which up to four molecules of CO can bind to a tetrameric Hb molecule (Williams et al., 1983). The affinity of HbCO for DPG is similar to that of HbO_2 (Labotka and Schwab, 1990). The site of the binding of DPG to tetrameric deoxyHb has been identified by x-ray crystallography to be the central cavity between the two β chains on the two-fold symmetry axis of the Hb molecule (Arnone, 1972). Although the site of binding in the liganded form of Hb (HbO_2 and HbCO) is still uncertain, it has been suggested to be close to that of deoxyHb (Gupta et al., 1979; Russu et al., 1990).

The interactions between Hb and DPG have been investigated extensively in model systems (Garby and deVerdier, 1971; Berger et al., 1973; Gerber et al., 1973; Hamasaki and Rose, 1974; Labotka and Schwab, 1990) but only one study, to our knowledge, has attempted to assess whether the dissociation constants (K_d) measured in model systems adequately describe the situation in intact erythrocytes (Marshall et al., 1977). In the latter ^{31}P nuclear magnetic resonance (NMR) study, the chemical shifts of the 3P and 2P resonances of DPG were shown to move to higher frequency when DPG binds to Hb. Hb solutions were used to define the relationship between the chemical shift changes and degree of binding of DPG to Hb, with the fraction of total DPG bound to Hb being quantified by using membrane ultrafiltration. By assuming that the linear relationship, which existed in solutions between the change in chemical shift and the measured fraction of the total DPG that was bound to Hb, could also be applied to intact erythrocytes, it was concluded that a larger fraction of the total DPG was bound to Hb in oxygenated and deoxygenated erythrocytes than in the corresponding solutions. However, the usefulness of this ^{31}P NMR method to investigate the interactions between DPG and Hb in intact erythrocytes is limited because the chemical shifts of the DPG resonances depend on several factors other

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than the extent of binding of DPG to Hb. These factors, which have been discussed by Labotka and Schwab (1990), include changes in magnetic susceptibility that occur with the change in redox state of the iron in the Hb, "solvent" effects that vary with Hb concentration, and the high pH dependence of the ^{31}P chemical shifts of the DPG resonances. In the present work we used pulsed-field gradient (PFG) NMR measurements of the diffusion coefficient (D) of DPG in both Hb solutions and in intact erythrocytes to study the affinity of Hb for DPG, and demonstrated a greater affinity of deoxyHb, compared with liganded Hb, for DPG in intact cells.

The theory underlying this relatively novel use of the diffusion measurement to measure molecular association is as follows. For unbounded diffusion of a molecular species in an isotropic medium, the signal intensity recorded by a PFG spin-echo (SE) experiment, normalized to the signal obtained in the absence of the magnetic field gradient pulses, R, is given by Stejskal and Tanner (1965).

$$R = \exp\left[-\gamma^2 g^2 \delta^2 D\left(\Delta - \frac{\delta}{3}\right)\right], \quad (1)$$

where g, δ, and Δ are the magnitude, duration, and time interval between the magnetic field gradient pulses, respectively, and γ is the magnetogyric ratio of the observed nucleus. In the presence of one or more boundaries such as cell membranes the motion of molecules is restricted by the boundary resulting in a mean net displacement in a given time, which is less than it would have been in the absence of the boundary (Latour et al., 1993). For molecules such as DPG that are confined to the interior of an erythrocyte, an apparent diffusion coefficient (D_{app}) can be estimated using Eq. 1. This value incorporates the effect that the cell membrane has on restricting the otherwise unimpeded motion of the molecule and is less than the value of the unrestricted (isotropic) diffusion coefficient, D_{ur}.

In solutions containing DPG and Hb the D of DPG, measured by a PFG experiment, can be expressed as the sum of the values of D of the free and bound forms, weighted by the number fraction of the two forms:

$$D = D_b P_b + D_f(1 - P_b), \quad (2)$$

where D_b and D_f are the values of D of the bound and free form of DPG, respectively, and P_b is the fraction of the total DPG population, which is bound to the Hb. The value of D_b is simply the D of Hb (D_{Hb}). Assuming that one molecule of DPG binds to one molecule of Hb (Hamasaki and Rose, 1974), then P_b can be expressed in terms of the dissociation constant, K_d, of the complex:

$$P_b = \frac{\mathcal{A}}{2[\mathrm{DPG}]_t}, \quad (3)$$

where

$$\mathcal{A} = [\mathrm{Hb}]_t + [\mathrm{DPG}]_t + K_d - \sqrt{([\mathrm{Hb}]_t + [\mathrm{DPG}]_t + K_d)^2 - 4[\mathrm{Hb}]_t[\mathrm{DPG}]_t}$$

where $[HB]_t$ and $[DPG]_t$ denote the total Hb and DPG concentrations, respectively, and K_d is given by

$$K_d = \frac{[\mathrm{Hb}][\mathrm{DPG}]}{[\mathrm{Hb\text{-}DPG}]}. \quad (4)$$

Thus, an estimate of K_d for an Hb complex with DPG can be derived using Eqs. 2–4 and a series of measurements of D of DPG obtained for different DPG concentrations, in the presence of a known Hb concentration. The objectives of this study were to estimate the values of K_d for DPG complexes with HbCO, HbO_2, and deoxyHb in solution and then to "test" whether these measurements of Hb affinity for DPG adequately described the dissociation of Hb-DPG complexes in intact erythrocytes. The latter objective was achieved by measuring the value of D_{app} of DPG in carbonmonoxygenated, oxygenated, and deoxygenated erythrocytes and then performing Monte Carlo (MC) simulations of diffusion (Lennon et al., 1994; Piton et al., 1994) in a biconcave disc (Beck, 1978) using values of D_{ur}, derived from the values of K_d estimated in the solutions, in the simulation.

MATERIALS AND METHODS

Materials

The DPG was obtained as the cyclohexylammonium salt (Grade 1, 99% purity), dysprosium chloride hexahydrate, tripolyphosphate pentasodium salt, hexahydrate (98% purity) and bis(2-hydroxyethyl)aminotris-(hydroxymethyl)-methane (bis-Tris) were from Sigma Chemical Co. (St. Louis, MO). Methylphosphonate (MeP) was from Aldrich (Milwaukee, WI), triethylphosphate (TEP) was obtained from BDH Chemicals Ltd. (Poole, UK). All other reagents were AR grade. The cyclohexylammonium salt of DPG was converted to the potassium form by passage through Dowex 50-H^+ (Fluka AG, Buchs, Germany) which had been previously washed with KCl (1 M) until the pH was constant (~6.0). The eluate was lyophilized, reconstituted in 100 mM bis-Tris/50 mM KCl buffer (pH 6.9), and kept at −4°C until required. The concentration of the stock DPG solution was determined, before freezing, from a fully relaxed ^{31}P NMR spectrum (intertransient delay 2 min) using a TEP capillary as a concentration reference. The T_1 of DPG in 150 mM NaCl was measured to be 12.50 ± 0.22 s (A. Xu, personal communication).

Hb concentration and pH

Hemoglobin concentrations were measured using one of the functions of a Sysmex CC-130 particle counter (Toa Medical Electronics, Kobe, Japan). The measurements were shown to be within 1% of the Hb concentration determined using the cyanomethemoglobin method ($\epsilon = 44\ mM^{-1}\ cm^{-1}$ for tetrameric Hb; van Kampen and Zijlstra, 1961). The Hb concentration inside erythrocytes was determined by measuring that in a lysed suspension of erythrocytes and dividing this value by the hematocrit (Ht) of the suspension. Hematocrits were measured in duplicate using a microhematocrit centrifuge (Clements, North Ryde, NSW, Australia) and a Hawkesley microhematocrit reader (Cope, London, U.K.). The measurements were multiplied by a factor of 0.97 to take into account the extracellular fluid trapped between the packed cells (Dacie and Lewis, 1975). The pHs of concentrated Hb solutions were measured using a glass-body combination pH probe with a calomel reference designed for use with protein solutions (Model No. AEP342, Activon Scientific Products, Thornleigh, NSW, Australia).

Hb solutions

Human erythrocyte suspensions obtained from the Red Cross Blood Transfusion Service (Sydney, NSW, Australia) were washed three times in four

volumes of 150 mM KCl (which had been gassed for 20 min with CO) and then packed by centrifugation (3000 × *g*, 5 min, 4°C). The supernatant was aspirated, and the packed cells were transferred to a separating funnel to which an equal volume of diethyl ether was added. The mixture was shaken vigorously to ensure complete lysis of the cells and maximum extraction of the membrane components into the ether phase, and then left to stand for 2 h at 4°C. The lower (aqueous) phase was isolated and filtered through Whatman No. 1 paper (Whatman, Ltd., Maidstone, U.K.) in a Buchner funnel (FSE, Strathfield, NSW, Australia). Residual ether was removed by rotary evaporation under reduced pressure (45 min, room temperature). The resulting (dilute) Hb solution was gently swirled in the presence of humidified CO to ensure CO saturation of the Hb and hence protection against the formation of methemoglobin (metHb, in which the Fe^{2+} is oxidized to Fe^{3+}). The solution was then concentrated overnight in an Amicon (Danvers, MA) ultrafiltration cell (Diaflo YM 10-membrane) at 4°C, under 50 mm Hg pressure provided by a nitrogen cylinder. The pH of the concentrated Hb solution (~6.5 mM) was generally in the range of 6.8–6.9; this was adjusted to 6.9 (if necessary) by dropwise addition of 1M NaOH, with stirring. The concentration of the Hb was determined, and then aliquots (5 ml) of the solution were placed in glass scintillation vials, saturated with humidified CO, and stored at −4°C until required (<1 month).

In experiments with HbCO, concentrated Hb aliquots were thawed, resaturated with humidified CO for 5 min while being swirled gently, and then kept at 4°C. Individual samples (1 ml) for the NMR diffusion experiments were prepared by adding concentrated COHb to a solution containing concentrated DPG, 100 mM bis-Tris/50 mM KCl buffer (pH 6.9) and a calibrated volume of 1 M HCl (to adjust the pH of the individual solutions to 6.9). TEP (0.3 M) and MeP (0.3 M) were added to each sample (final concentration 4 mM) as an NMR internal chemical shift reference, and pH reference, respectively (see NMR Methods).

The experiments using HbO_2 and deoxyHb were performed within a single NMR session. Concentrated Hb (20 ml) was thawed and transferred to a conical flask which exposed a large surface area of solution. The CO was removed from the Hb by exposing the solution to humidified O_2 under a 250-W (12-V) quartz-halogen spotlight at 4°C. The degassing process was accelerated by placing the flask on a rocker (to ensure adequate mixing of the Hb) and in a cabinet lined with aluminum foil (to maximize the light directed onto the sample). The percentages of remaining HbCO and metHb were estimated periodically using the spectrophotometric methods of van Kampen and Zijlstra (1965). After 40 h the HbCO content had been reduced from 90% to <1%. The metHb level remained at <4%. Aliquots (5 ml) of the resulting HbO_2 solution were transferred to glass scintillation vials, saturated with O_2, and stored at 4°C until required. Individual NMR samples (1.5 ml) were prepared from the stock HbO_2, as for the COHb samples, except that EDTA was added (final concentration 0.5 mM) to chelate any paramagnetic impurities that may have been present. This was necessary, because in preliminary experiments the ^{31}P NMR linewidths were intolerably large, and we surmised that this was due to the release of small amounts of Fe^{3+} from the Hb. The samples were prepared under humidified O_2, and after the HbO_2 sample had been loaded into the NMR diffusion cell (see NMR Methods), humidified O_2 was layered above the sample before the cell was capped and sealed with Parafilm (American Can Company, Greenwich, CT). The remaining solution was gassed with humidified N_2 for 20 min in a rotating tonometer to form deoxyHb. The latter solution was then transferred to an NMR diffusion cell, and humidified N_2 was layered above the sample in the cell. The percentage of metHb was measured for all samples and found to be less than 5%. Final Hb concentrations in the NMR samples were determined after the experiment.

Erythrocyte suspensions

Freshly drawn venous blood from a single donor (A.J.L.) was diluted into two volumes of ice-cold 150 mM NaCl and centrifuged (3000 × *g*, 5 min, 4°C). The diluted plasma and buffy coat were aspirated and the cells were then centrifugally washed three times more. For suspensions in which elevated levels of DPG were not required, two final washes were performed in washing medium (10 mM inosine, 10 mM pyruvate, 2.5 mM NaH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 5 mM TEP, 5 mM MeP, and 75 mM NaCl; pH 7.4; osmolality adjusted to 310 mOsm kg^{-1} with addition of NaCl) before the cells were packed to an Ht of ~0.80 and then loaded into an NMR diffusion cell.

Carbonmonoxygenation of the cells was carried out by saturating the washing medium with CO for 20 min before use and bubbling humidified CO through the suspension for 5 min before the final centrifugation step. Deoxygenation of cell suspensions was carried out by gassing the final suspension (Ht ~ 0.80) with humidified N_2 in the rotating tonometer for 20 min. Dysprosium tripolyphosphate ($Dy(PPP)_2^{7-}$) was added, in a final concentration of 3 mM in the extracellular volume, to the deoxygenated cell suspensions to balance the magnetic susceptibility between the inside of the cells and the extracellular fluid (Fabry and San George, 1983).

Levels of DPG were raised in carbonmonoxygenated cells by using an adaptation of the method of Petersen et al. (1990). The cells were washed twice in incubation medium (10 mM inosine; 10 mM pyruvate, 50 mM NaH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 5 mM TEP, 5 mM MeP, 75 mM NaCl; pH 7.4) and then incubated in the medium at 37°C at an Ht of ~0.20. At various times between 3 and 10 h of incubation, aliquots of the total incubation suspension were centrifuged, washed twice in washing medium, packed to an Ht of ~0.80, and loaded into NMR diffusion cells.

Erythrocyte suspensions in which the intracellular pH was increased or decreased were obtained by washing the suspensions four times in incubation medium, the pH of which had been adjusted to give a desired intracellular value. Each wash consisted of centrifuging the suspension (3000 × *g*, 5 min, 4°C), aspirating the supernatant, adding incubation medium (adjusted to the desired pH) so that the Ht was ~0.20, and then incubating the suspension at 37°C for 5 min. The cells, once equilibrated at the desired pH, were washed twice in washing medium, the pH of which had been adjusted to the desired value, before being loaded into NMR diffusion cells.

NMR methods

The spectrometer was a Bruker AMX 400 (Bruker, Karlsruhe, Germany) with a wide-bore 9.4-T superconducting magnet (Oxford Instruments, Oxford, UK). The operating frequency for ^{31}P was 161.9 MHz, and the Fourier transform mode was used for spectral processing. The linear power-amplifier for the PFG apparatus (Bruker) was under software control. The magnetic field gradients were generated by an actively shielded coil assembly mounted in fiberglas/epoxy resin around the radiofrequency (rf) coils of a wide-bore multinuclear (15–162 MHz) probe, designed for 10-mm outside diameter sample tubes. They were applied in the same direction as the field from the spectrometer magnet ($\mathbf{B_0}$). The magnitude of the magnetic field gradient, calibrated from PFG analysis using the known D of H_2O at 25°C (Mills, 1973), was 47.5 ± 0.3 T $m^{-1}A^{-1}$. Samples for PFG experiments were placed in a cylindrical semimicro bulb (10-mm outer tube; volume ~0.5 ml; outside cylinder height 10 mm; inside cylinder height 7.5 mm; Cat. 529-E-10; Wilmad, Buena, NJ), which was subsequently inserted into a 10-mm NMR tube containing 2.5 ml of CCl_4.

The PFG longitudinal eddy-current delay (LED) pulse sequence (Gibbs and Johnson, 1991), which is based on the stimulated echo (STE) pulse sequence (Hahn, 1950; Tanner, 1970), was used to measure the D of DPG in the Hb solutions. The pulse sequence was modified by the incorporation of further homospoil gradient pulses after the second and fourth rf pulses (Fig. 1 *B*; Waldeck et al., 1993). Adjustments to the duration of the second magnetic field gradient pulse were required to compensate for mismatches between the area under the two gradient pulses (Haner and Schleich, 1989); the magnitude of these adjustments was estimated as described by Kuchel and Chapman (1991). The relationship between R and the parameters of a PFGLED experiment is the same as that for a PFGSE experiment (i.e., Eq. 1). The PFGSE pulse sequence (Fig. 1 *A*, Stejskal and Tanner, 1965) was used in experiments involving cell suspensions, because this achieved the maximum possible signal-to-noise ratio; the echo intensity in an STE experiment is diminished by a factor of 0.5 from that possible in an SE experiment (Stilbs, 1987). In all experiments, a series of spectra were recorded with g being incremented from one spectrum to the next, while δ and Δ were maintained constant.

Composite-pulse ^1H WALTZ-16 decoupling (Shaka et al., 1983) was applied continuously during the experiments to remove the proton-

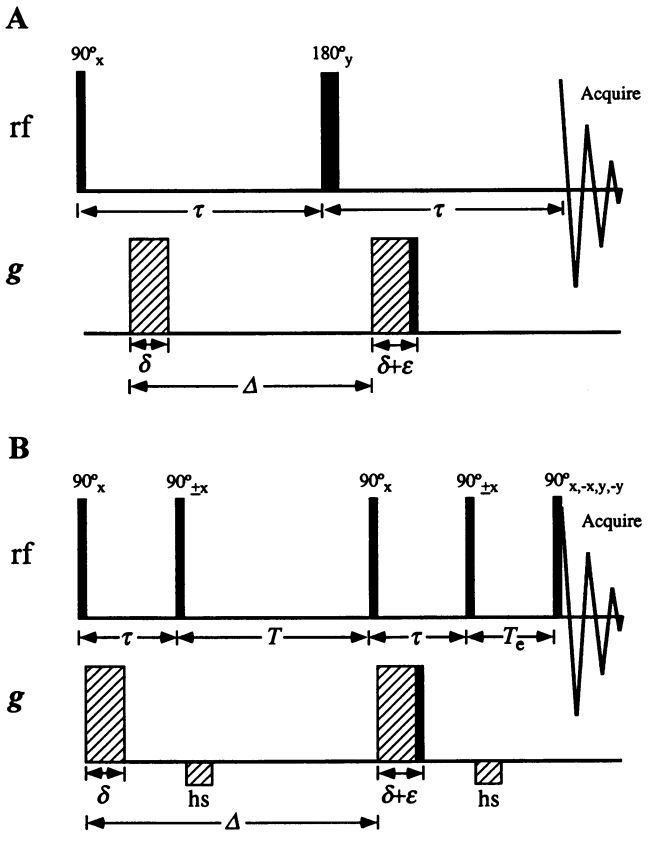

FIGURE 1 NMR pulse sequences used for measuring translational diffusion coefficients of molecules. The PFGSE (Stejskal and Tanner, 1965) and a modified form of the PFGLED (Gibbs and Johnson, 1991; Waldeck et al., 1993) pulse sequences are depicted in (*A*) and (*B*), respectively. In both pulse sequences any imbalance between the time integrals of the two magnetic field gradient pulses was compensated for by an experimentally determined correction factor, ϵ (Kuchel and Chapman, 1991). The PFGSE sequence was used in experiments involving cell suspensions ($\tau = \Delta = 20$ ms; $\delta = 14$ ms). The PFGLED pulse sequence was employed in experiments involving Hb solutions ($\tau = 15$ ms; $T = 20$ ms; $\delta = 12$ ms; $T_e = 20$ ms). The PFGLED pulse sequence included negative homospoil (hs) gradient pulses (~120 mT m^{-1}; 3 ms) to eliminate any transverse magnetization that may have arisen during the longitudinal storage periods (T and T_e). The rf pulses, directly after the field gradient pulses, were phase cycled to cancel out contributions from the longitudinal magnetization to the echo.

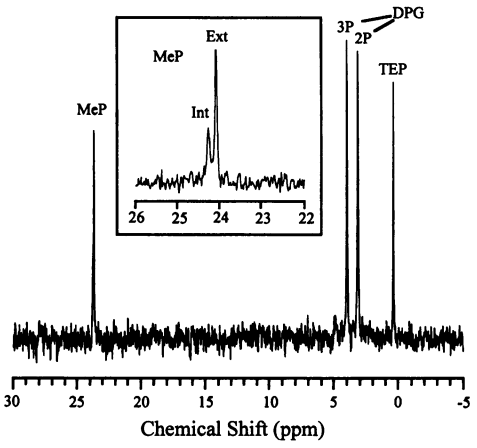

FIGURE 2 ^{31}P NMR SE spectrum of a carbonmonoxygenated erythrocyte suspension. The delay between the 90 and 180° rf pulses, τ (Fig. 1), was 20 ms. The spectrum was derived from 128 transients (intertransient delay, 3 s) of spectral width 10 kHz and processed into 16K data points, using an exponential line-broadening factor of 3 Hz. The erythrocytes were packed to an Ht of 0.82 and the Hb and DPG intracellular concentrations (mM) were 5.7 and 6.2, respectively. The intracellular pH was estimated to be 7.28 from the chemical shift of the intracellular MeP resonance (23.757 ppm) which, in this spectrum, is almost coincident with the extracellular MeP resonance. The internal and external MeP resonances were more clearly separated in suspensions that had been suspended in incubation medium for more than 3 h (see *inset*); in the example shown the intra- and extracellular pH values were estimated to be 6.95 and 7.04, respectively. Abbreviations: MeP, methylphosphonate [intracellular(int) and extracellular(ext)]; DPG, 2,3-bisphosphoglycerate (3P and 2P resonances); TEP, triethylphosphate.

phosphorus coupling; this did not cause any adverse phase-modulation effects with this particular molecular system. The sample heating caused by the decoupling was estimated to be 2°C; this was done by recording a ^{1}H NMR spectrum of ethylene glycol (Bubb et al., 1988) before and after a PFGLED (and PFGSE) experiment in which composite-pulse decoupling parameters were the same as those used in the experiments involving Hb solutions or cell suspensions. Hence, the temperature control on the spectrometer was set to 23°C for all PFG experiments, thus ensuring that the diffusion measurements were made at 25 ± 1°C.

In general, spectra were recorded for 16 different g values; however, for the very low DPG concentrations, the increased number of transients required to achieve a signal-to-noise ratio that enabled analysis necessitated fewer spectra (usually eight) being acquired. All spectra were derived from 16-K data points and a spectral width of 10 kHz; exponential multiplication of the free induction decay by a line-broadening factor of 3 Hz was performed in all cases (Fig. 2). The number of transients recorded depended on the DPG concentration in the sample. For each sample a "dummy" SE or LED spectrum ($g = 0$ T m^{-1}) was acquired before the PFG experiment to ensure that the sample had thermally equilibrated. A further, similar, spectrum was acquired immediately after the experiment to determine whether any hydrolysis of DPG had occurred during the PFG experiment. A final fully relaxed ^{31}P spectrum (intertransient delay 90 s) was recorded to quantify the DPG concentration by using the TEP (4 mM) as an intensity standard (Fig. 2); DPG is 100% NMR visible in ^{31}P NMR spectra of erythrocytes (Petersen et al., 1990), and so its concentration (mM) was able to be estimated from the ratio of the integral of the 3P resonance of DPG to that of the TEP resonance. For experiments involving cell suspensions this calculated concentration of DPG was then divided by the Ht to obtain the intracellular concentration of DPG. The DPG concentrations in Hb solutions, estimated from the fully relaxed spectra, agreed to within 5% of the value expected from the gravimetric procedures used in sample preparation.

The TEP also acted as an internal frequency reference (0.44 ppm to high frequency of the 85% phosphoric acid resonance; Kirk et al., 1986). The pH of the sample was estimated from the chemical shift of added (titratable) MeP (4 mM; Fig. 2) and a calibration curve performed at 25°C using the method of Stewart et al. (1986). The apparent pK_a of the MeP and the chemical shifts of the acidic and basic forms were estimated to be 7.431 ± 0.016, 25.100 ± 0.005, and 21.61 ± 0.05, respectively. The pH of the sample was estimated from the mean of the chemical shift value of MeP in the spectra acquired before and after the PFG experiment. In erythrocyte suspensions the intra- and extracellular pH was estimated from the separate resonances arising from the two MeP populations, respectively. (Stewart et al., 1986). Changes in pH during the course of a PFG experiment were generally minimal and did not exceed 0.05.

Analysis of PFG experiments

The D of DPG was estimated from nonlinear least squares regression (NLLS; Osborne, 1976) of Eq. 1 onto the amplitudes of the 3P and the 2P (where it was resolved) resonances of DPG (Fig. 2). In some experiments the amplitude of the 2P resonance was affected by the adjacent inorganic

phosphate resonance and thus was not used in the analysis. Where data from both resonances were analyzed the D of DPG was expressed as the weighted mean of the estimates arising from the individual resonances. The control spectrum that was acquired after the PFG experiment was used to ascertain whether significant hydrolysis of DPG had occurred during the experiment. If the dimunition in peak amplitudes exceeded 5%, then a linear rate of hydrolysis was assumed, and peak amplitudes from the PFG spectra were corrected accordingly. The overall extent of hydrolysis never exceeded 15%, and hydrolysis was only evident in experiments that either involved cell suspensions or exceeded 3 h in duration (i.e., low-DPG concentrations). As stated above, diffusion within an erythrocyte is restricted by the presence of the cell membrane and therefore the values of D obtained by NLLS regression of Eq. 1 onto experimental data obtained from cell suspensions represented apparent values, viz D_{app}.

Estimates of the value of K_d from the experiments involving Hb solutions were obtained by weighted (by the reciprocal of the variance of the individual D values) NLLS regression of Eqs. 2–4 onto the estimates of D of DPG, measured as a function of DPG concentration. The parameters, [Hb] and D_{Hb}, were taken to be constants in the regression. The latter parameter was set to $(1.01 \pm 0.06) \times 10^{-11}$ m^{2}s^{-1} for experiments where the Hb concentration was ~5 mM (Kuchel and Chapman, 1991); for lower Hb concentrations the value of D_{Hb} was measured in a separate PFGSE experiment using the method of Kuchel and Chapman (1991).

Monte Carlo simulations of diffusion

The diffusion of DPG in an erythrocyte, as detected by a PFGSE NMR experiment, was simulated by performing a three-dimensional random walk for an ensemble of particles within a biconcave disc. In the simulations Δ and δ were fixed values, and R was recorded as a function of increasing g ($n = 8$). The biconcave disc shape of a human erythrocyte can be approximated by a degree-four (quartic) surface called a cyclide (Kuchel et al., 1987). In Cartesian coordinates it has the form (Moon and Spencer, 1988):

$$(x^2 + y^2 + z^2)^2 - 2E(x^2 + y^2) - 2Fz^2 + G = 0, \quad (5)$$

where E, F, and G are constants that are chosen ("fitted") to give conformity to the shape that characterizes an erythrocytic biconcave disc (i.e., its diameter and maximum and minimum thickness); in all the simulations the latter characteristic dimensions were set to 8.0, 2.5, and 1.0 μm, respectively (Beck, 1978; see Fig. 3 and Appendix A).

The random walk consisted of steps of a nominated, fixed length, s, with a random (± 1) direction in each of the three Cartesian dimensions. The time, t, taken for each step, was

$$t = \frac{s^2}{2D}, \quad (6)$$

and the number of steps, N, performed for each particle (for each value of g) was

$$N = \frac{(\Delta + \delta)}{t}. \quad (7)$$

Step lengths were selected as described by Lennon and Kuchel (1994). The simulations were repeated for 10,000 independent, DPG point-molecules (particles). The random walk for each particle was commenced at a different (random) starting location within the biconcave disc.

Erythrocytes have been reported to become oriented (Higashi et al., 1993), at least under some conditions (e.g., Ht < 0.01), in magnetic fields of magnitudes used in this study (i.e., 9.4 T). Consequently the MC simulations of diffusion were performed both in biconcave discs (Eq. 5) that were randomly oriented with respect to **g** (and $\mathbf{B_0}$) and in discs that where aligned with their disc plane parallel to **g** (Higashi et al., 1993). In the simulations **g** was set to an azimuthal angle, θ, between 0 and $\pi/2$, relative to the z-axis of the Cartesian coordinate system. Random selections of the latter angle enabled the random orientations of erythrocytes in a suspension to be simulated. Steps in the random walk were performed along an axis that was set to be colinear with **g** (z') and a corresponding set of orthogonal axes (x and y'; Fig. 3). The position of the particle (in Cartesian coordinates) was then computed and the surface (boundary) conditions enforced using Eq. 5. After a step was completed, the projection of the final displacement vector of the particle onto **g** was computed ($y \sin\theta + z \cos\theta$) and converted to a change in phase of the magnetization vector, by multiplication with the factor $\gamma g t$ (Carr and Purcell, 1954). The phase changes for individual steps were added during the first τ interval andsubtracted during the second τ interval (Carr and Purcell, 1954). The contribution of each particle to the total signal (for a particular value of **g**) was computed as the cosine of the total phase change of the particle multiplied by the factor, $\sin\theta$, to weight each angle by its probability density within a sphere (Callaghan, 1991). Erythrocytes oriented with their disc planes parallel to **g** were simulated by setting θ to $\pi/2$ for all particles.

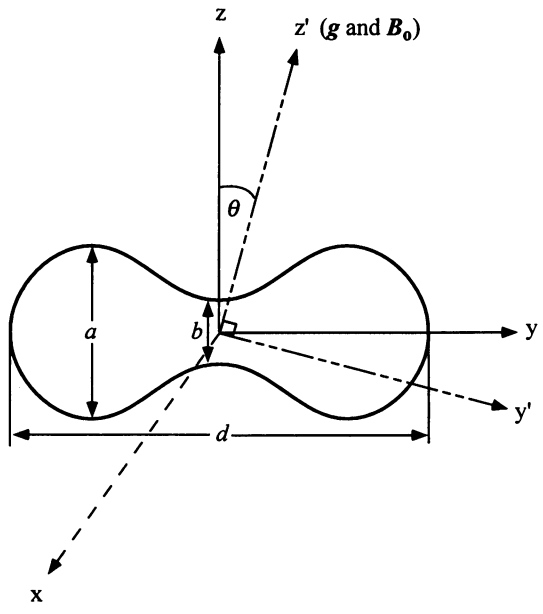

FIGURE 3 The biconcave disc model used in the MC simulations of diffusion in erythrocytes. The surface of the biconcave disc was described in a Cartesian coordinate system by Eq. 5 and the parameters E, F, and G were derived from the characteristic dimensions a, b, and d which were set to 8.0, 2.5, and 1.0 μm, respectively (Beck, 1978), for all simulations. Random packing of erythrocytes in suspensions were simulated by setting the direction of the axis of the applied magnetic field gradient (z') to a (random) azimuthal angle, θ, between 0 and $\pi/2$, relative to the z-axis of the Cartesian coordinate system. After random steps of magnitude $\sqrt{2\,Dt}$ (Eq. 6), were performed along the x, y' and z' axes, the final position of a particle (point-molecule) was computed in Cartesian coordinates and the Cartesian coordinate surface (boundary) condition (Eq. 5) was enforced.

Reflection of a particle at the plasma membrane was simulated by placing the particle, which had encountered the surface during a step, on the sphere surface at the point of intersection of the step vector and the surface (Appendix B). The fraction of the step thus completed was computed, and random steps of the remaining step length were performed until a step resulted in the particle being returned to the interior of the cell. The random number generators used in the simulations have been described elsewhere (Lennon and Kuchel, 1994). The simulation programs were written in the "C" programming language, compiled using Microsoft Visual C (Version 1.0), and run on a 33-MHz 486-DX microcomputer.

RESULTS

Dissociation constants estimated from Hb solutions

Dissociation constants (mM) for the binding of DPG to HbCO, HbO_2, and deoxyHb in solutions containing Hb and

DPG (pH 6.9; 25°C; 100 mM bis-Tris/50 mM KCl) were estimated to be 1.98 ± 0.26, 1.8 ± 0.5, and 0.39 ± 0.26, respectively (Fig. 4). These values indicate a 4.5-fold increase in affinity of Hb for DPG on deoxygenation. Other known physiological effector molecules, such as Mg^{2+} and ATP, were absent in this study because both the DPG and ATP would have been depleted with storage of the erythrocytes (Bock et al., 1985) before the preparation of the Hb solution and the Mg^{2+} would have been largely removed in the ultrafiltration process.

To ascertain whether the values of K_d estimated using this method varied significantly with the concentration of Hb (in the range 2.5–5.0 mM), the HbCO experiment was repeated using an Hb concentration of 2.7 mM. The value of K_d estimated from that experiment, 1.5 ± 0.8 mM, suggested that the affinity of HbCO for DPG does not alter significantly over the Hb concentration range of 2.7–5.0 mM. It was concluded that the different Hb concentrations employed in the experiments detailed in Fig. 4 would not have significantly affected the values of K_d estimated when due consideration was given to the errors in the individual measurements. The use of different Hb concentrations in these experiments enabled a relationship to be established between the D_f and Hb concentration. This relationship was found to be linear over an Hb concentration range of 2.7–5.0 mM ($D_f = a + b$[Hb]; $a = 4.319 \pm 0.029\ m^2s^{-1}$; $b = -0.511 \pm 0.007\ m^2s^{-1}mM^{-1}$).

Measurements of the D_{app} of DPG in erythrocyte suspensions

The D_{app} of DPG in erythrocyte suspensions with Hb in the deoxy state was significantly lower than that with the Hb in the carbonmonoxy or oxy state (Fig. 5; Table 1). To determine whether this difference in D_{app} was the result of the $Dys(PPP)_2^{7-}$ that was present in the extracellular volume of the deoxygenated erythrocyte suspensions, D_{app} was estimated in a suspension that was not gassed and in the presence and absence of $Dys(PPP)_2^{7-}$. The estimates of D_{app} ($10^{11} \times m^2s^{-1}$) were 4.2 ± 0.4 and 4.5 ± 0.29 in the absence and in the presence of $Dys(PPP)_2^{7-}$, respectively. This result implied that addition of $Dys(PPP)_2^{7-}$ did not affect the rate of diffusion of DPG within the erythrocytes. The values of D_{app} measured in carbonmonoxygenated, oxygenated, and deoxygenated suspensions suggest that differences in the affinity of the liganded and deoxy forms of Hb for DPG are indeed manifest at a cellular level. Given the difficulty incurred in maintaining all experimental variables (pH, etc.) constant, except for the ligand-state of Hb, the difference between the D_{app} values estimated in erythrocytes having Hb in the carbonmonoxy and oxy form was considered insignificant; the weighted mean of these values was $(6.17 \pm 0.20) \times 10^{-11}\ m^2s^{-1}$.

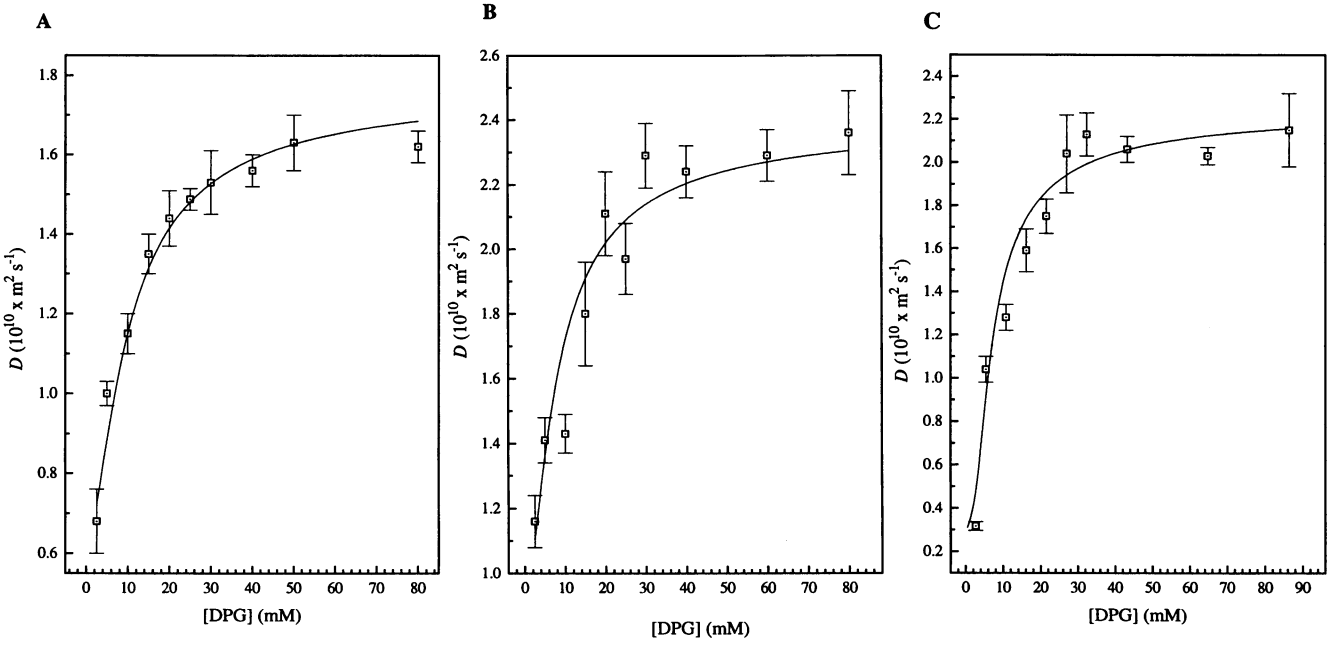

FIGURE 4 The diffusion coefficient of DPG in carbonmonoxy- (*A*), oxy- (*B*) and deoxy- (*C*) Hb solutions as a function of DPG concentration. Each value of *D* was derived from a PFGLED NMR experiment conducted at 25°C; the error bars denote the error in the estimated value of *D* obtained from NLLS regression of Eq. 1 onto the amplitudes of the DPG resonances recorded in the NMR experiment. Estimates of K_d and D_f for each of the Hb ligand states were obtained by performing (weighted) NLLS regression of Eqs. 2–4 onto the estimated values of *D*. The K_d values (mM) thus estimated were 1.98 ± 0.26, 1.8 ± 0.5, and 0.39 ± 0.26 for carbonmonoxy-, oxy-, and deoxyHB, respectively. The values of D_f ($10^{10} \times m^2s^{-1}$) were estimated to be 1.787 ± 0.023, 2.41 ± 0.05, and 2.26 ± 0.05 for HbCO, HbO_2 and deoxyHB, respectively. The latter values reflected the Hb concentrations (mM) used for the experiments; viz 4.96 ± 0.05, 3.75 ± 0.09 and 4.0 ± 0.2, respectively. In the regression the D_{Hb} ($10^{11} \times m^2s^{-1}$) was taken to be 1.01 ± 0.06 (Kuchel and Chapman, 1991). The latter value was measured in a hemolysate in which the concentration of Hb was 4.65 mM. The mean pH (±SD), estimated from the chemical shift of the MeP resonance in the spectra ($g = 0\ T\ m^{-1}$) recorded immediately before and after the PFGLED experiment, for each of the HbCO, HbO_2, and deoxyHb solutions was 6.85 ± 0.05, 6.90 ± 0.10, and 6.98 ± 0.15, respectively.

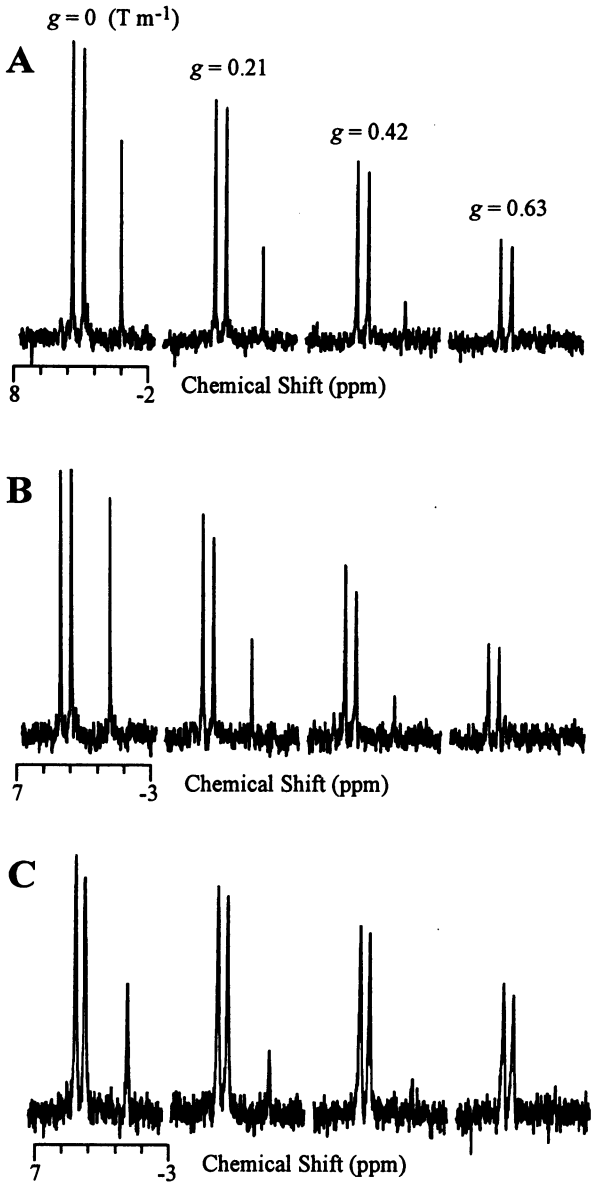

FIGURE 5 Spectra from typical PFGSE experiments conducted in erythrocyte suspensions having Hb in the carbonmonoxy (*A*), oxy (*B*), and deoxy (*C*) form. Four out of a total of 16 spectra are shown for each experiment. The spectra from (*A*) and (*B*) were derived from 128 transients; the poorer signal-to-noise ratio in the spectra from the deoxygenated cells (*C*) necessitated the accumulation of 256 transients/spectrum. The intertransient delay was 3 s, the spectral width was 10 kHz, and the free induction decays were processed into 16K data points using an exponential line-broadening factor of 3 Hz for all spectra. Before acquisition of the spectra, four dummy transients were performed to equilibrate the magnetization in the sample. The PFGSE parameters were: $\tau = \Delta = 20$ ms; $\delta = 14$ ms; and g_{inc} ($n = 16$) = 52.5 mT m^{-1}. The Ht, Hb, and DPG intracellular concentrations (mM) were, respectively, 0.82, 5.7, and 6.2 for (*A*); 0.76, 5.6, and 6.0 for (*B*); and 0.76, 5.7, and 6.5 for (*C*). The intracellular pH, estimated from the mean values of the chemical shift of the intracellular MeP resonance, recorded immediately before and after the experiment, was 7.22, 7.30, and 7.38 for suspensions (*A*), (*B*), and (*C*), respectively. The linewidth of the 3P resonance was measured to be 10, 11, and 27 Hz for (*A*), (*B*), and (*C*), respectively. The linewidths of the 3P and 2P resonances did not vary significantly (generally <1%) in the carbonmonoxygenated and oxygenated suspensions (e.g., *A* and *B*), however for deoxygenated cells (e.g., *C*) the linewidths of DPG resonances sometimes increased by ~3 Hz in the spectra recorded with high magnetic field gradient strengths.

TABLE 1 Diffusion coefficients of DPG in human erythrocytes estimated using suspensions at 25 °C.

Hemoglobin ligand-state	Number of Experiments	pH*	D‡ ($10^{11} \times m^2 s^{-1}$)
Carbonmonoxy	3	7.25 ± 0.03	5.89 ± 0.18
Oxy	2	7.27 ± 0.04	6.66 ± 0.24
Deoxy	2	7.38 ± 0.02	4.10 ± 0.23

The erythrocytes were from a single donor (A.J.L.) and were suspended (Ht ~0.80) in washing medium (pH 7.4). Dysprosium tripolyphosphate (3 mM in the extracellular volume) was added to the deoxygenated cell suspension to balance the magnetic susceptibility inside and outside of the cells. The mean (± SD) intracellular Hb and DPG concentrations in the suspensions were 5.70 ± 0.05 and 6.6 ± 0.7 mM, respectively. PFG parameters used are as detailed in the caption of Fig. 5.

* Values of pH were estimated from the chemical shift of the intracellular MeP resonance (Stewart et al., 1986).

‡ The estimated value of *D* represents the weighted mean of the estimates arising from the analysis of the attenuation (Eq. 1) of the 3P and 2P resonances of DPG.

We surmised that elevating the concentration of DPG in the erythrocytes (through preincubation in the medium containing inosine and pyruvate) would result in a smaller fraction of the total DPG within the cell being bound to the Hb, and that this would be reflected in a larger D_{app}. This, however, was not the case; D_{app} did not alter significantly over the DPG concentration range 5–55 mM in carbonmonoxygenated erythrocyte suspensions (Fig. 6). Similarly, changes in the intracellular pH over the range 6.6–7.75 in deoxygenated erythrocytes did not result in appreciable changes in the value of D_{app} (Fig. 7).

Even though the diffusion time used for the PFGSE experiments was reasonably short (~20 ms) the average motion of those molecules confined to the interior of the cell would have been impeded to some extent by the cell membrane (Latour et al., 1993). To examine whether the values of K_d estimated in the Hb solutions could adequately predict the D_{app} of DPG in intact erythrocytes, values of D_{ur} (where *ur* denotes unrestricted) for DPG, were interpolated for the same Hb and DPG concentrations measured in the cell suspensions (viz, 5.7 mM and 6.6 mM, respectively) by using a (linear) extrapolation to estimate the value of D_f. The values of D_{ur} ($10^{11} \times m^2 s^{-1}$), 7.2 and 5.0 for HbO_2 and deoxyHb, respectively, were used in Eq. 6 for MC simulations of diffusion in randomly oriented biconcave discs and in discs in which θ was set to $\pi/2$ (see Materials and Methods). The enforcement of the surface condition (i.e., Eq. 5) by the simulation program was checked by plotting the *x*, *y*, and *z* coordinate trajectories of a set of ~50 particles (Fig. 8) during a single transient of a spectrum in a PFGSE experiment. The confinement of the absolute values of the *x*, *y*, and *z* coordinate positions of the particles to ≤4, ≤4, and ≤1.25 μm, respectively, showed that the surface condition was satisfactorily enforced. Also the absence of any discontinuities in the trajectories implied that the points of intersection between step vectors and the surface were being calculated correctly (see Appendix B). The method of simulating randomly oriented biconcave discs was tested by performing a

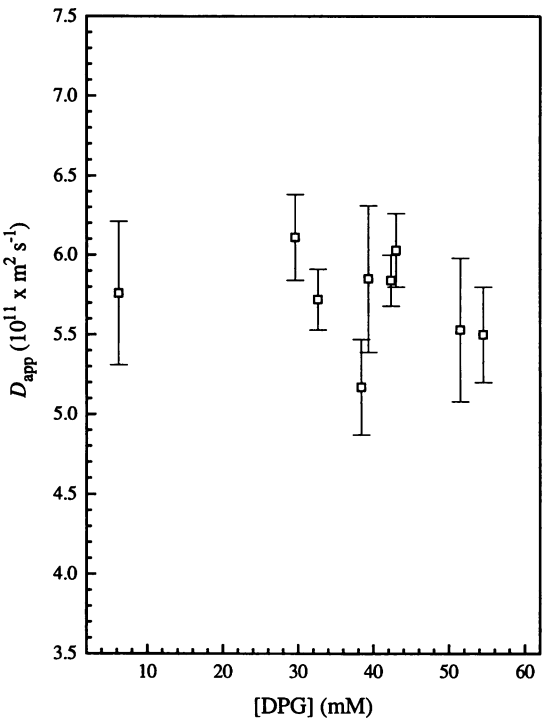

FIGURE 6 The D_{app} of DPG measured as a function of DPG concentration in erythrocytes which had been suspended at 37°C in incubation medium for between 3 and 10 h. Each measurement was the result of a PFGSE NMR experiment employing 16 different values of g and conducted at 25°C. The values of D were estimated from the attenuation of the 3P resonance of DPG, given that incubation generally resulted in a large intracellular inorganic phosphate resonance which artifactually affected the amplitude of the neighboring 2P resonance. The DPG concentrations were determined from "fully relaxed" spectra (intertransient delay 90 s) recorded after the PFGSE experiments. The mean intracellular pH, estimated from the mean values of the chemical shift of the intracellular MeP, resonance recorded immediately before and after the experiment, was 6.90 ± 0.04.

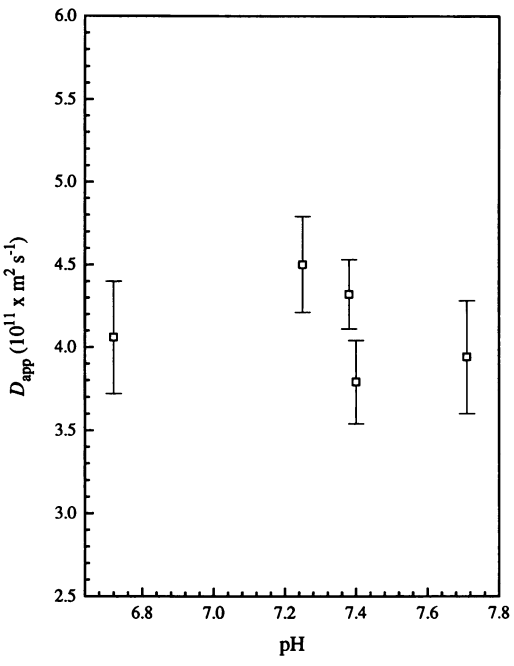

FIGURE 7 The D_{app} of DPG measured as a function of pH in erythrocytes, which had been washed four times with pH-adjusted incubation medium. Each measurement was the result of a PFGSE NMR experiment employing 16 different values of g and conducted at 25°C. The pH of the samples used for each measurement, estimated from the chemical shift of the intracellular MeP resonance, was the mean of the pH estimated immediately before and after the PFGSE NMR experiment.

simulation in which the surface condition was ignored (i.e., free diffusion). Analysis of these simulated values of R resulted in an estimate of D (Eq. 1), which was within 1% of the value of D (Eq. 6) actually used in the simulation.

Simulated values of D_{app} were estimated by performing NLLS regression of Eq. 1 onto the simulated (normalized) signal intensities. The values, thus obtained using randomly oriented and aligned (i.e., $\theta = \pi/2$) biconcave discs, were all less than the experimental values for oxygenated and deoxygenated cells (Table 2); the reasons for this are considered in the Discussion.

To examine the relative affinity of HbO_2 and deoxyHb for DPG in erythrocytes, the ratio of the values of D_{app} for deoxygenated and oxygenated erythrocytes, $D_{app,deoxy}/D_{app,oxy}$, was computed for the simulated and experimental values of D_{app} (Table 2). The ratios obtained from the MC simulations, 0.80 ± 0.05 and 0.74 ± 0.05, for random and aligned erythrocyte orientations, respectively, were both higher than the experimental value of 0.62 ± 0.06. MC simulations were also performed using values of D_{ur} for DPG estimated using the same parameters and the values of K_d reported for deoxyHb and HbO_2 complexes with DPG by Berger et al. (1973) and Hamasaki and Rose (1974); the results of these simulations are also detailed in Table 2.

The alignment of biconcave discs with their disc plane parallel to **g** (i.e., $\theta = \pi/2$) results in the DPG molecules experiencing less restriction of their motion along the z' axis (see Fig. 3) than they would experience with other orientations. Therefore the simulated values of D_{app} were greater when θ was set to $\pi/2$ than when θ was randomly assigned a value between 0 and $\pi/2$. Furthermore the extent of restriction was reduced more on alignment (i.e., $\theta = \pi/2$) in oxygenated than in deoxygenated cells because a larger fraction of the total DPG population of molecules encountered the surface (boundary) during the simulated PFGSE experiment in the former cells. This resulted in the value of the ratio, $D_{app,deoxy}/D_{app,oxy}$, being less when $\theta = \pi/2$ compared with the case when random values of θ were used.

DISCUSSION

Estimates of K_d obtained from solutions: HbCO and HbO_2

The estimates of K_d were, in general, consistent with those made using other methods. The value of K_d estimated here for HbCO in model solutions (1.98 ± 0.26 mM) is slightly less than the value of 2.4 mM measured by Labotka and Schwab (1990) using an equilibrium dialysis method. Although both measurements were performed at pH 6.9 the

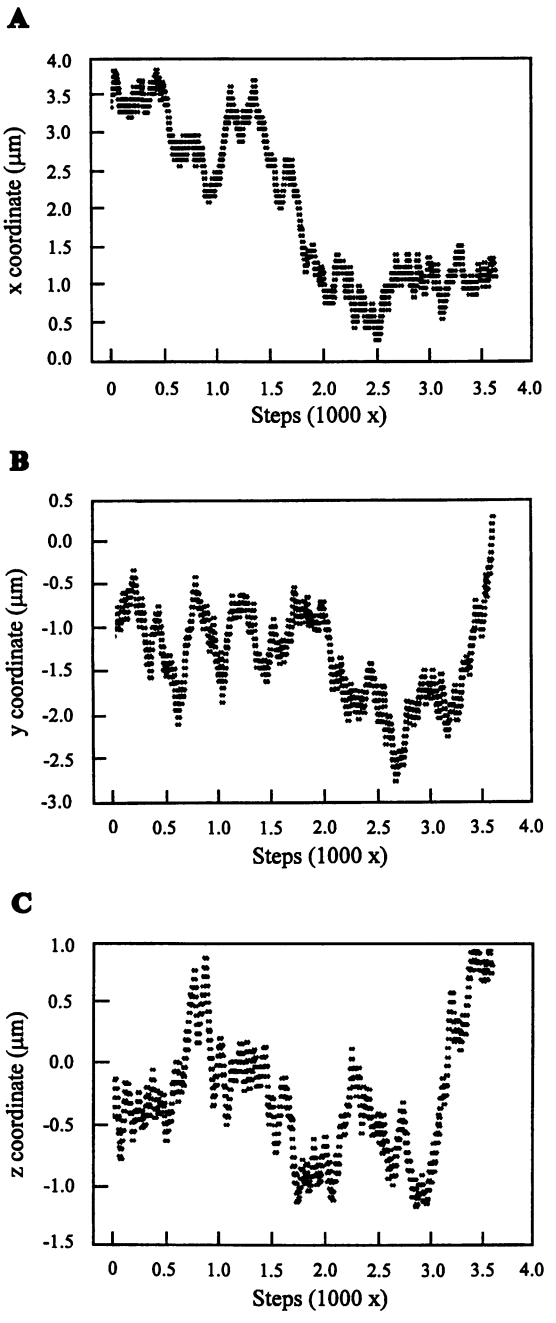

FIGURE 8 Typical particle trajectory recorded from an MC simulation of a random walk in a biconcave disc (Fig. 3) oriented with its axis of symmetry along the z axis. The trajectory ((*A*), x coordinate; (*B*), y coordinate; and (*C*), z coordinate) confirmed that the particle was indeed confined by the boundary conditions and that a continuous path was followed by the particle. The dimensions of the model erythrocyte (biconcave disc) were: $d = 8$ μm; $a = 2.5$ μm, and $b = 1.0$ μm. Other parameters used in the simulation were: $D = 8.4 \times 10^{-11}$ m^{2}s^{-1}; $g = 0$ mT m^{-1}; $\Delta = 20$ ms; $\delta = 14$ ms and $s = 0.04$ μm. The x, y, and z coordinate positions of a single particle and a single value of g, at each of the $N = 3570$ random steps (Eqs. 6 and 7) that were performed, are plotted in (*A*), (*B*), and (*C*), respectively, as a function of step number. The simulation trajectories, for a definable number of particles, were stored in a "history" file during the MC simulation, and then later used as input for a "C" program which visualized the data on the screen of a 33 MHz 486 computer in the format of the above figures. The visualization program was compiled using Microsoft Visual C (V1.0) and the screen images produced were captured using the screen dump facilities of Microsoft Windows (V3.1) and stored as encapsulated postscript files.

latter was made at 21°C in the presence of a higher chloride ion concentration (100 mM KCl/24 mM bis-Tris) and a lower Hb concentration (2.8 mM compared with the 4.96 mM used in this study). The reasonable agreement that exists between these two estimates, bearing in mind the differences in experimental conditions employed, suggests that the method detailed in this study can be used, in general, to investigate the interaction between a macromolecule and a ligand when the value of K_d for the interaction is in the millimolar range. It is not strictly valid to compare our estimates of the binding affinity of HbO_2 for DPG with previously reported estimates because of the large differences in the experimental conditions under which the measurements were made.

Previous measurements of K_d for HbO_2 and DPG, performed under similar experimental conditions (i.e., in 0.1–0.12 M Cl^-, pH 7.2–7.3, and Hb concentrations ranging from 0.05 to 4 mM) and summarized by Hamasaki and Rose (1974), are in reasonable agreement with the value of 2.5 mM at 20–25°C; the value of K_d estimated in this study (1.8 ± 0.5 mM) was close to this "consensus" value. The similar values of K_d measured here for HbCO and HbO_2 suggest that the structural details of the interactions between both liganded forms of Hb and DPG are similar.

DeoxyHb

The estimates of K_d made here were much more at variance with previous studies than was the case for HbCO and HbO_2. Furthermore, values of K_d recorded in the literature for deoxyHb and DPG under similar conditions (Hamasaki and Rose, 1974) have not yielded a "consensus" value. Measurements by Berger et al. (1973) conducted at 37°C ($K_d = 2 \times 10^{-4}$ M) imply that the affinity of deoxyHb for DPG is 25 times that of HbO_2 at 25°C (calculated using a value of $\Delta H = 46$ kJ mol^{-1} for the dissociation reaction; Hamasaki and Rose, 1974). Hamasaki and Rose (1974), using measurements made at 25°C ($K_d = 4.22 \times 10^{-5}$ M), estimated an increase of ~60–fold in affinity on deoxygenation of Hb. Our results suggested that the affinity of the Hb was increased by a factor of ~4.5 on deoxygenation ($K_d = 0.39 \pm 0.26$ mM); therefore, this value is considerably lower than in the previous studies.

The inherent insensitivity of the NMR phenomenon necessitates the use of many transients per spectrum for low concentrations of DPG and thus our experiments extended over a long time (e.g., 5 h when the DPG concentration was 2.5 mM). This resulted in some hydrolysis of DPG (always <10% in solutions) and small pH changes (always <0.05). These factors contribute to a general difficulty in obtaining reliable data at low-ligand concentrations, and small differences in the estimates of D (at low-ligand concentrations) can result in large differences in the estimate of K_d. In contrast, because the values of K_d measured for HbCO and HbO_2 were in the mM range the dissociation curves (Fig. 4) were not so dependent on the estimates of D at low concentrations of DPG.

TABLE 2 Values of D_{app} of DPG in deoxygenated and oxygenated human erythrocytes obtained by MC simulations

D values	MC-simulated using K_d values from this study*	MC-simulated using K_d values from Berger et al. (1973)‡	MC-simulated using K_d values from Hamasaki and Rose (1974)§	Experimental
D_{ur} ($10^{11} \times m^2 s^{-1}$)				
Deoxy	5.00 ± 0.20¶	3.00 ± 0.20	1.60 ± 0.20	
Oxy	7.20 ± 0.20	7.10 ± 0.20	7.10 ± 0.20	
D_{app} ($10^{11} \times m^2 s^{-1}$)				
Deoxy				4.10 ± 0.23
Random	2.81 ± 0.11	2.00 ± 0.13	1.20 ± 0.15	
$\theta = \pi/2$	3.60 ± 0.14	2.39 ± 0.16	1.33 ± 0.17	
Oxy				6.66 ± 0.24
Random	3.51 ± 0.10	3.45 ± 0.10	3.45 ± 0.10	
$\theta = \pi/2$	4.87 ± 0.14	4.71 ± 0.13	4.71 ± 0.13	
$D_{app,deoxy}/D_{app,oxy}$				0.62 ± 0.06
Random	0.80 ± 0.05	0.58 ± 0.05	0.35 ± 0.05	
$\theta = \pi/2$	0.74 ± 0.05	0.51 ± 0.05	0.28 ± 0.04	

The simulated values of D_{app} were obtained by performing MC simulations of diffusion within randomly oriented biconcave discs having a diameter (d) and maximum (a) and minimum (b) thickness of 8.0, 2.5, and 1.0 μm, respectively (Fig. 3). The PFGSE parameters used for both the MC simulations and the PFG experiments were as detailed in Fig. 5 with the exception that 16 spectra were recorded in the PFG experiments (g_{inc} = 52.5 mT m^{-1}) and only eight different g values were used in the MC simulations (g_{inc} = 105 mT m^{-1}). The values of D_{ur} that were used for the MC simulations were obtained from simulated graphs in which the D of DPG was plotted as a function of the DPG concentration using the parameters: $D_b = 1.01 \times 10^{-11}$ m^2 s^{-1} (Kuchel and Chapman, 1991); $D_f = 1.4 \times 10^{-10}$ m^2 s^{-1}; $[Hb]_t$ = 5.7 mM; and values of K_d were as indicated in the table. Values of D_{ur} corresponding to a $[DPG]_t$ = 6.6 mM were estimated from these graphs. The particular concentrations of DPG and Hb used for the simulations were selected because they represented the mean values of those measured in the erythrocyte suspensions that were used to obtain the experimental values of D_{app}.

* The values of K_d (mM) used were as measured in Hb solutions in this study (i.e., 0.39 ± 0.26 and 1.8 ± 0.5 for deoxyHb and HbO_2, respectively).

‡ Berger et al. (1973) measured the values of K_d at 37°C. These values (0.2 mM and 4 mM for deoxyHb and HbO_2, respectively), adjusted for a temperature of 25°C using enthalpy values (kJ mol^{-1}) for the dissociation reactions (46 and 28 for deoxyHb and HbO_2, respectively; Hamasaki and Rose, 1974), were 97 μM and 2.5 mM for deoxyHb and HbO_2, respectively.

§ The values of K_d used were as measured by Hamasaki and Rose (1974; i.e., 42 μM and 2.5 mM for deoxyHb and HbO_2, respectively).

¶ The error in the value of D_{ur} was estimated to be 0.2×10^{-11} m^2 s^{-1} for all cases; this value represented the uncertainty in reading the values from the simulated binding curves. Because the error in the estimate of D_{app}, obtained by NLLS regression of Eq. 1 onto the simulated signal intensities was in all cases <1%, the error in the value of D_{app} was assumed to be dominated by that in D_{ur}.

Another potential source of error in K_d was the value of the D_{Hb} used in the analysis. This value, 1.01×10^{-11} $m^2 s^{-1}$ (Kuchel and Chapman, 1991), was measured in a hemolysate with an Hb concentration of 4.65 mM; the concentrations of the Hb (mM) in the present solutions were 4.96, 3.75, and 4.0 for HbCO, HBO_2 and deoxyHb, respectively. Given that the P_b of the DPG was very much less in the liganded-Hb solutions, the term P_bD_b in Eq. 2 would have been $\ll (1 - P_b)D_f$, and so errors in D_b (i.e., D_{Hb}) would not have been so critical in these analyses. This error could be reduced by obtaining a well defined relationship between the D_{Hb} and Hb concentration.

Dependence of the value of K_d on Hb concentration

Garby and De Verdier (1971) claimed that the affinity of both deoxyHb and HbO_2 for DPG decreased markedly as the concentration of Hb was increased from 0.4 to 5.5 mM. This finding contrasts with that of Hamasaki and Rose (1974) who reported that the affinity of Hb for DPG was independent of the concentration of Hb in the range 0.06–4 mM. In our study we found no significant difference in the value of K_d for the HbCO complex with DPG estimated at the two Hb concentrations 2.7 and 4.96 mM. We assumed in the present analysis that the value of K_d does not vary appreciably with Hb concentration in the range 3.5–5.7 mM, but further studies are necessary to define better the relationship between Hb concentration and the affinity of the various forms of Hb for DPG. The value of K_d might be expected to depend on the concentration of the Hb, because the Hb molecules may occupy a significant fraction of the total sample volume, and therefore the concentration of the DPG in the available volume will be higher than if its concentration is quoted relative to the total volume. For example, in 1 ml of a 5-mM Hb solution [M_r = 64,500 (Dacie and Lewis, 1975), partial specific volume of 0.74 ml g^{-1} (Cohn and Edsall, 1943)], the excluded volume is ~240 μl. When the reduced volume available to the DPG molecules in this solution is accounted for, the effective concentration of DPG, originally quoted as 5 mM in the total volume, is 6.6 mM. Also, in solutions having high concentrations of Hb (~5 mM), interactions between Hb molecules may obscure some potential binding sites for DPG. These are but two of the more obvious phenomena that render the Hb-DPG solutions thermodynamically non-ideal, and could thus alter the apparent value of K_d.

Affinity of Hb for DPG in intact erythrocytes

The MC simulations of diffusion in biconcave discs, using the values of K_d measured in the Hb solutions, yielded ratios of $D_{app,deoxy}/D_{app,oxy}$ equal to 0.80 ± 0.05 and 0.74 ± 0.05, for randomly oriented and aligned ($\theta = \pi/2$) biconcave discs, respectively; these ratios are higher than the ratio observed in intact erythrocytes (0.62 ± 0.06; Table 2). The ratios that were obtained from simulations using the values of K_d

estimated by Berger et al. (1973; 0.58 ± 0.05 and 0.51 ± 0.05 for randomly oriented and aligned discs, respectively) were, however, much closer to the experimental ratio, whereas use of the values of K_d estimated by Hamasaki and Rose (1974) resulted in ratios (0.35 ± 0.05 and 0.28, respectively), which were much lower than we observed experimentally. This method of examining whether the values of K_d measured in model solutions are consistent with measurements of D of DPG obtained from intact erythrocytes does not, however, account for the presence of other effector ions or molecules, such as Mg^{2+} and ATP, which were absent in the Hb solutions and yet were present in the erythrocytes. The total ATP and Mg^{2+} concentrations in intact erythrocytes are 1.35 mM (Beutler and Mathai, 1967) and 3.06 mM (Herring et al., 1960), respectively. The interactions between Mg^{2+}, ATP, DPG and deoxyHb, and HbO_2 are complex; they, of course, depend on the relative concentrations of the species present and the dissociation constants of the various complexes formed. Notwithstanding this complexity Berger et al. (1973) measured association constants (at pH 7.2, 37°C) for the relevant complexes (Table 3) and these values were then used by Gerber et al. (1973) to estimate the distribution of the various species under simulated physiological conditions (pH 7.2; 37°C; 130 mM KCl; 20 mM NaCl). From figures contained in the original publication (Gerber et al., 1973), the P_b values of DPG in solutions which contain Hb, DPG, ATP, and Mg^{2+} at concentrations (mM) of 6.7, 6.0, 2.0, and 2.8, respectively, are 0.54 and 0.87 for HbO_2 and deoxyHb, respectively. Substitution of these values into Eq. 3 yielded apparent values of K_d (mM) of 0.22 and 2.9 for deoxyHb and HbO_2, respectively. The values of K_d (mM) measured in the absence of Mg^{2+} and ATP, under the experimental conditions used by Gerber et al. (1973), were 0.2 and 4.0 for deoxyHb and HbO_2, respectively. This suggests that, at least under the experimental conditions adopted in the abovementioned study, the presence of Mg^{2+} and ATP at physiological concentrations results in an increase in the apparent affinity of HbO_2 for DPG and a small decrease in the apparent affinity of deoxyHb for DPG.

Incorporating these effects (of Mg^{2+} and ATP) into the MC simulations, at least qualitatively, would be manifest in a (slightly) larger value of $D_{app,deoxy}/D_{app,oxy}$ for the MC simulations. This further supports our conclusion that the value of K_d, measured for the deoxyHb complex with DPG in this study is, in fact, an overestimate and that the estimates of K_d reported by Berger et al. (1973) appear to describe the relative affinities of deoxyHb and HbO_2 for DPG adequately within intact erythrocytes. A more straightforward method of accounting for the effect of other erythrocyte metabolites on the interaction between DPG and Hb would be to estimate the values of K_d in Hb solutions containing the physiological concentrations of each effector. Other potentially significant effectors such as bicarbonate and inorganic phosphate (Gerber et al., 1973) could also be included.

TABLE 3 Dissociation constants for complexes which form between deoxyHb, HbO_2, DPG, ATP, and Mg^{2+} as measured by Berger et al. (1973) and used in the calculations of Gerber et al. (1973)

Interaction	K_d (mM) deoxyHb	HbO_2
Hb + DPG	0.20	4.0
Hb + ATP	0.38	2.8
Hb + MgATP	7.1	25.6
Mg^{2+} + ATP	0.083	
Mg^{2+} + DPG	1.67	

Measurements, quoted as association constants in the original publications, were performed at pH 7.2, 37°C in 130 mM KCl/20 mM NaCl.

Incubating carbonmonoxygenated erythrocytes in a medium containing inosine and pyruvate incubation medium results in increased levels of DPG while the ATP remains at physiological levels (Petersen et al., 1990). The concomitant accumulation of organic phosphates results in a Donnan-mediated lowering of the intracellular pH (the mean pH in the incubated erythrocytes was 6.90 ± 0.04; Fig. 2, inset). Therefore, it is likely that the affinity of the HbCO for the DPG would have been increased by this lowered pH. However, at very high DPG concentrations (up to 55 mM) a large fraction of the total DPG would not have been complexed to the HbCO if one-to-one binding occurred. No increase in the value of D_{app} was experimentally observed in the suspensions with elevated DPG levels; in fact, with very high concentrations of DPG there appeared to be a slight decrease in the value of D_{app} (Fig. 6).

Fossel and Solomon (1976) postulated that DPG interacts with the cell membrane of dog erythrocytes and is linked to Na^+ transport in those cells. In human erythrocytes, DPG has been shown to increase the lateral diffusion of integral membrane proteins when added to leaky membrane systems (Schindler et al., 1980), weaken associations in the cytoskeletal complex (Sheetz and Casaly, 1980; Cohen and Foley, 1984), and affect the fragility and deformability of the membrane (Chasis and Mohandas, 1986). Moriyama et al. (1993) reported two major sites of attachment of the membrane skeleton to the bilayer that are specifically dissociated by physiological concentrations of DPG. These findings all suggest that DPG interacts with sites in the cytoskeleton and on the cell membrane at physiological concentrations, and therefore, at the elevated concentrations of DPG realized in this study, a larger proportion of those sites could be occupied by DPG. This would have resulted in a third population of DPG molecules that were practically immobile during the time scale of the PFG NMR experiment. The existence of this putative fraction of the total DPG pool is consistent with the observation that the D_{app} of DPG did not increase when the concentration of DPG inside the erythrocyte was elevated with respect to physiological levels.

The affinities of HbO_2 and deoxyHb for DPG in model solutions at 37°C have been shown to decrease when the pH is increased from 7.1 to 7.7 with the change in affinity being greatest for deoxyHb (Garby and De Verdier, 1971). This effect was not manifest in the values of D_{app} measured in deoxygenated erythrocyte suspensions in which the intracellular pH was manipulated to lie between 6.8 and 7.7 (Fig. 7). Changes in pH may alter the affinity of different sites for

DPG in different ways, therefore it is difficult to anticipate the manner in which the value of D_{app} would alter with these changes. Also, changes in the charge of the diffusing species resulting from pH changes may alter their values of D_{ur} in the cytoplasm (Bell, 1964; Wolynes, 1980). Consequently, because of the multiple indirect effects that changes in the intracellular pH may have had on the value of D_{app} of DPG, these PFG NMR measurements must be interpreted cautiously.

MC simulations

The values of D_{app} estimated in erythrocyte suspensions were higher than the corresponding values yielded by the MC simulations of diffusion in biconcave discs which were either randomly orientated or aligned such that their disc plane was parallel with **g** (and $\mathbf{B}_0$); this presents an interesting difference upon which we can, presently, only speculate. These results suggest that even if the magnetic field had an orienting effect on the erythrocytes in the suspensions used for this work, this effect alone does not account for the difference between simulated and experimental values of D_{app} of DPG in erythrocytes. Indeed the existence of this effect is controversial. Researchers in this laboratory did not observe the effect, subsequently reported by Higashi et al. (1993), when they exposed erythrocyte suspensions (Ht ~0.05) to magnetic fields of 9.4 T and used 0.5% low-melting-temperature agarose to fix the cells (P. W. Kuchel and B. E. Chapman, unpublished results). Furthermore, the extent to which erythrocytes could be oriented in high-Ht suspensions is unknown.

Other speculated causes of the difference between the simulated and experimental values of D_{app} include the possibility that other biconcave disc dimensions or other shapes, such as rectangular prisms, may more accurately simulate the surface condition (e.g., Simpson, 1993). Also, it is possible that the assumption that diffusion within the cell is described by a three-dimensional random walk in a homogeneous medium is not accurate. Failure of this condition would imply that the value of D_f estimated from the Hb solutions, is a poor estimate of that parameter within intact erythrocytes. Therefore, because this latter value defines the asymptotic value of D_{ur} for DPG, the values of D_{ur} estimated from physiological concentrations of Hb and DPG could have been very different.

There has been conjecture that movement of metabolites within cells is facilitated by the phenomenon of "reduced dimensionality" (Adam and Delbrück, 1968). Surfaces within the intracellular architecture can effectively reduce the volume through which a molecule is constrained to move to its next position, thus resulting in translational motion that is faster than predicted for a three-dimensional walk. This could be investigated by making comparisons between simulated and experimental values of D_{app} for other erythrocyte metabolites. The sensitivity of the MC simulation to different cell shapes could also be studied.

In the meantime, however, if it is assumed that a comparison of the experimental ratio, $D_{app,deoxy}/D_{app,oxy}$, measured in this study (Table 2, column 5), and simulated ratios (Table 2, columns 2–4) represents a reasonable method of "testing" whether values of K_d measured in solution are useful for describing the dissociation of Hb-DPG complexes in intact cells, then the measurements made by Berger et al. (1973) at 37°C and adjusted to 25°C describe best the relative affinities of deoxyHb and HbO_2 in intact erythrocytes.

SUMMARY AND CONCLUSIONS

We have demonstrated that PFG NMR measurements of the value of D of a ligand in the presence of a macromolecule to which it binds can be used to estimate the value of K_d of the complex. This could also apply to multisite binding with the appropriate modifications made to account for the changes in D of the ligand (and, if appropriate, D of the macromolecule) when complexes were formed. The use of this method to measure reliably the affinity of a ligand for a macromolecule is probably limited at this stage to those systems in which the value of K_d lies in the millimolar range; this is because of the difficulty in detecting the signals from very low (μM) concentrations of ligand in PFG NMR spectra.

An important advantage of the PFG technique is its ability to measure mobilities of molecules within intact cells. The next stage in the analysis, MC simulations, can be used to evaluate the extent to which the value of D_{ur} is affected by the confinement imparted by the membrane. The values of D_{app} thus estimated can then be compared with those measured from cell suspensions.

APPENDIX: MATHEMATICAL ASPECTS OF THE MC SIMULATIONS

A. Parameters defining the shape of the biconcave disc

The parameters E, F, and G in Eq. 5 can be expressed in terms of the dimensions of a biconcave disc, viz, the diameter of the maximum circular cross-section, d, and the maximum and minimum thickness a and b, respectively (see Fig. 3). These terms are

$$E = \frac{d^2}{4} - \frac{a^2}{4}\left(\frac{d^2}{b^2} - 1\right)\left[1 - \sqrt{1 - \frac{b^2}{a^2}}\right] \quad \text{(A1)}$$

$$F = \frac{d^2}{b^2}E - \frac{b^2}{8}\left(\frac{d^4}{b^4} - 1\right) \quad \text{(A2)}$$

$$G = \frac{d^2}{2}E - \frac{d^4}{16} \quad \text{(A3)}$$

B. Aspects of a random walk in a biconcave disc

Determining whether a particle is located outside of the erythrocyte

In a given random step a particle (or point molecule) moves from a position P (x_1, y_1, z_1), which is inside the simulated cell (biconcave disc) centered on (0, 0, 0), to a position Q (x_2, y_2, z_2). To determine whether the position Q is located outside the cell, the point A (x, y, z) is defined as the point of intersection between the displacement vector $\overrightarrow{OQ}$ and the surface of the cell

(i.e., Eq. 5). The vector, $\overrightarrow{OA}$ is defined as,

$$\overrightarrow{OA} = \frac{t(x_2\mathbf{i} + y_2\mathbf{j} + z_2\mathbf{k})}{m} \tag{B1}$$

where m and t denote the magnitude of $\overrightarrow{OQ}$ and the relative position of A along $\overrightarrow{OQ}$, respectively. Substituting the Cartesian coordinates of the point A (i.e., (tx_2/m), (ty_2/m), (tz_2/m)) into Eq. 5 yields a quadratic in t^2:

$$\frac{t^4[x^4 + y^4 + z^4 + 2x^2y^2 + 2x^2z^2 + 2y^2z^2]}{m^4} - \frac{t^2[2E(x^2 + z^2) + 2Fz^2]}{m^2} + G = 0. \tag{B2}$$

If, $|t| \leq m$, then the point Q lies inside the cell; otherwise the particle has moved outside of the cell, and this condition requires the coordinates of the point of intersection between the step vector (i.e., $\overrightarrow{PQ}$) and the surface of the erythrocyte (Eq. 5) to be computed.

Computing the point of intersection between the step vector and the cell surface

If the particle has moved out of the erythrocyte in a step (see Appendix B above) then the point of intersection of the step vector (i.e., $\overrightarrow{PQ}$) with the erythrocyte surface is defined as B(x, y, z). The vector, $\overrightarrow{OB}$, is defined as

$$\overrightarrow{OB} = \overrightarrow{OP} + \frac{t\overrightarrow{PQ}}{n}, \tag{B3}$$

where n and t denote the magnitude of $\overrightarrow{PQ}$, and the relative position of B along $\overrightarrow{PQ}$, respectively. Substitution of the Cartesian coordinates of the point B (i.e., $x_1 + t(x_2 - x_1)/n$, $y_1 + t(y_2 - y_1)/n$, $z_1 + t(z_2 - z_1)/n$) into Eq. 5 results in a quartic expression in t:

$$\begin{aligned}
&\frac{t^4}{n^4}[\Delta x^4 + \Delta y^4 + \Delta z^4 + 2(\Delta x^2\Delta y^2 + \Delta x^2\Delta z^2 + \Delta y^2\Delta z^2)] \\
&+ \frac{t^3}{n^3}\left[4\begin{pmatrix} x_1(\Delta x)^3 + y_1(\Delta y)^3 + z_1(\Delta z)^3 + x_1\Delta x\Delta y^2 + y_1\Delta x^2\Delta y \\ + x_1\Delta x\Delta z^2 + z_1\Delta x^2\Delta z + y_1\Delta y\Delta z^2 + z_1\Delta y^2\Delta z \end{pmatrix}\right] \\
&+ \frac{t^2}{n^2}\left[\begin{matrix} 6(x_1^2\Delta x^2 + y_1^2\Delta y^2 + z_1^2\Delta z^2) \\ + 2\begin{pmatrix} x_1^2\Delta y^2 + 4x_1y_1\Delta x\Delta y + y_1^2\Delta x^2 + x_1^2\Delta z^2 \\ + 4x_1z_1\Delta x\Delta z + z_1^2\Delta x^2 + y_1^2\Delta z^2 + 4y_1z_1\Delta y\Delta z \\ + z_1^2\Delta y^2 - E(\Delta x^2 + \Delta y^2) - F\Delta z^2 \end{pmatrix}\end{matrix}\right] \\
&+ \frac{t}{n}\left[4\begin{pmatrix} x_1^3\Delta x + y_1^3\Delta y + z_1^3\Delta z + x_1^2y_1\Delta y + x_1y_1^2\Delta x \\ + x_1^2z_1\Delta z + x_1z_1^2\Delta x + y_1^2z_1\Delta z + y_1z_1^2\Delta y \\ - E(x_1\Delta x + y_1\Delta y) - Fz_1\Delta z \end{pmatrix}\right] \\
&+ [x_1^4 + y_1^4 + z_1^4 + 2x_1^2y_1^2 + 2x_1^2z_1^2 + 2y_1^2z_1^2 - 2E(x_1^2 + y_1^2) - 2Fz_1^2 + G] \\
&= 0
\end{aligned} \tag{B4}$$

where Δx, Δy, and Δz denote $(x_2 - x_1)$, $(y_2 - y_1)$ and $(z_2 - z_1)$, respectively. Values for t were obtained in the MC simulations by using Ferrari's method to solve the quartic equation, Eq. B4 (Turnbull, 1957). Cartesian coordinates for the point B were obtained from Eq. B3.

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