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The effect of cadmium ions on 2,3-bisphosphoglycerate in erythrocytes studied with ^{31}P NMR

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The interaction of cadmium ions with human red blood cell (RBC) 2,3-bisphosphoglycerate (DPG) was studied by ^{31}P NMR. ^{31}P NMR spectra and ^{31}P T_1 and ^{31}P T_2 relaxation times give evidence for cadmium–2,3-bisphosphoglycerate complexation in aqueous solution. ^{31}P NMR spectra indicate the occurrence of a similar complexation in RBC cryolysates. The lag phase (constant polyphosphate level) prior to DPG hydrolysis in incubated red blood cells is lengthened in the presence of cadmium.

^{31}P relaxation; ^{31}P-NMR; Cadmium-bisphosphoglycerate interaction; Erythrocyte

1. INTRODUCTION

^{31}P NMR spectroscopy has been used in various studies to examine metabolic processes of intact normal and diseased erythrocytes [1–8]. An advantage of this method is the nondestructive observation of the red blood cells (RBCs) over an incubation period. Additional information can be gained from the ^{31}P spin-lattice relaxation time (T_1) and the ^{31}P spin-spin relaxation time (T_2). Rabenstein et al. [9] have described the interactions between Cd^{2+} and RBC glutathione and hemoglobin, respectively. Here, we employ ^{31}P NMR to investigate free polyphosphates in RBCs and their interaction with monitoring NMR spectra and T_1 and T_2 relaxation times.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

RBCs obtained from freshly collected heparinized blood were washed at 37°C three to four times with equal volumes of isotonic 0.1 M Bis-Tris buffer (pH 7.4) containing saline and 5 mM glucose (medium A) in D_2O. For ^{31}P NMR measurements 9 vol. packed RBCs were suspended in 1 vol. medium A with and without 10 mM $CdCl_2$ (final concentration 1 mM). Bacterial growth was prevented by addition of infusion solution containing 2% (v/v) ampicillin (10 mg/ml), refobacin (0.8 mg/ml).

RBC cryolysates were prepared using the freeze-thaw technique with packed erythrocytes, which had been washed three to four times with medium A (in H_2O). The cryolysates were stored in liquid N_2 prior to the NMR measurements. The NMR measurements were performed with cryolysates suspended in an equal volume of 0.1 M Bis-Tris buffer, pH 7.4 (in D_2O), with and without 2 mM $CdCl_2$ (final cadmium concentration 1 mM).

DPG (Sigma, St. Louis) as the pentacyclohexylammonium salt was used. DPG was dissolved in 0.1 M Bis-Tris buffer, pH 7.4 (in D_2O), either without or with $CdCl_2$ in a DPG/$CdCl_2$ molar ratio of 5:1. For T_1 measurements a DPG/$CdCl_2$ molar ratio of 1:1 was used at pH 6.6. The pH of all solutions was determined prior to the NMR measurements and was not corrected for the isotope effect.

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2.2. *NMR measurements and data analysis*

^{31}P spectra and ^{31}P relaxation data were measured at 109 MHz on an AM 270 FT spectrometer (Bruker, Karlsruhe). Spectra of RBC cryolysates and aqueous DPG solutions were measured in 10 mm diameter NMR tubes spinning in a high-resolution probe head at 25°C. Spectra of intact RBCs were recorded in 15 mm diameter tubes, not spun, using a high-resolution probe head at 37°C. The FIDs were collected with 8K data points and a spectral width of 3, 5, and 10 kHz for DPG solutions, cryolysates and RBCs, respectively.

^{31}P relaxation data were collected at 15°C and at 121 MHz on a CXP 300 FT spectrometer (Bruker, Karlsruhe). All samples (only aqueous solutions of DPG were degassed) were measured in 10 mM NMR tubes, not spun, in a high-power probe head. An inversion recovery pulse sequence of 20 data points with each point consisting of 20 FIDs of 8K data points was employed for T_1 determinations. A CPMG pulse sequence sampling 150–200 echoes from an approximate total of 4000 and spread over the full range of the magnetization decay curve was employed for T_2 determinations. A delay time of 20–30 between the high-power pulse trains was used avoiding heating of samples.

All ppm values are given relative to the external standard of 85% D_3PO_4. For T_1, a monoexponential least-squares fit was employed for each resonance of the ^{31}P spectra. For T_2, both a monoexponential fit and a multiexponential analysis were performed. The multiexponential functions arising from summation of individual relaxation curves attributed to different phosphorus classes are decomposed by employing the 'peeling off' method after semi-logarithmic linearization followed by the eigenfunction expansion analysis. This analysis is described in detail elsewhere [10,11]. For the incubation study of RBCs three independent integrations of the DPG signals and of that of P_i were averaged. These intensity values were then calculated as percent of the total ^{31}P signal strength of the RBC spectra to be plotted vs duration of incubation.

3. RESULTS AND DISCUSSION

3.1. *Relaxation times*

Table 1 lists T_1 and T_2 relaxation times of the two phosphate resonances of DPG, in solutions and RBC cryolysates. The spin-lattice relaxation time T_1 of the 3-P and 2-P resonances of DPG in RBC cryolysates is approximately half that of the respective relaxation times of DPG in aqueous solutions. Multiexponential analysis of T_2 decay curves of total phosphorus shows the existence of only one component for DPG in aqueous solutions and in RBC cryolysates. The T_2 value of total phosphate in cryolysates increases with the breakdown of DPG to P_i from 160 ms (approx. 2:1 molar ratio of DPG/P_i as determined from the NMR spectrum) to 230 ms (approx. 1:1 molar ratio of DPG/P_i. These data imply that P_i has a larger T_2 value, resulting in an increase in the overall T_2 of the aged cryolysate. This observation is in full agreement with T_2 relaxation time studies of DPG and P_i solutions, where DPG was observed to have the shorter T_2 relaxation time [12]. Despite this slow time-dependent increase in T_2 of cryolysates, it is still substantially smaller than the T_2 of aqueous DPG. The decrease in T_1 and T_2 relaxation times of DPG in cryolysates is most likely due to the greater viscosity of the cryolysates and to paramagnetic effects from dissolved dioxygen in the RBC cryolysates.

The T_1 relaxation times of both phosphate resonances of DPG decrease by approx. 40% upon addition of an equimolar concentration in Cd^{2+}, indicating DPG-Cd^{2+} complexation. The T_2 relax-

Table 1

The effect of Cd^{2+} on ^{31}P T_1 and T_2 relaxation times of DPG and P_i, in aqueous solutions and RBC cryolysates

Sample	T_1 (s)[a]			T_2 (s)[b]
	3-P	2-P	P_i	Total phosphorus
DPG	3.40	2.70	-	0.527
DPG + $CdCl_2$	2.14	1.65	-	0.145
Cryolysate (fresh)	1.36	1.42	1.03	0.160
Cryolysate (5 h old)	-	-	-	0.230

[a] 100 mM DPG, dissolved in 0.1 M Bis-Tris buffer, pH 6.6 (in D_2O), with and without 100 mM $CdCl_2$, or cryolysate of washed packed RBCs with and without 100 mM $CdCl_2$

[b] 5 mM DPG, dissolved in 0.1 M Bis-Tris buffer, pH 7.4 (in H_2O), with and without 10 mM $CdCl_2$, or cryolysate of washed packed RBCs with and without 10 mM $CdCl_2$

All measurements are taken at 15°C. Standard deviations are typically $\leq 5\%$

ation time of aqueous DPG decreases by approx. 70% upon addition of Cd^{2+}, also confirming Cd^{2+} binding. It appears that there is a small increase in the T_2 relaxation time of RBC cryolysates in the presence of Cd^{2+} but this change is difficult to interpret in view of the slowly increasing T_2 relaxation times of the cryolysate in the absence of Cd^{2+}.

3.2. *^{31}P NMR spectra*

The spectrum of RBC cryolysates shows down-field shifts of both phosphate resonances of DPG when compared to the respective aqueous solution spectrum of DPG (see fig.1). Dilution experiments with cryolysates indicate that this down-field shift decreases with dilution. Addition of Cd^{2+} results in down-field shifts of the 3-phosphate resonance of approx. 0.25 and 0.07 ppm and of the 2-phosphate resonance of 0.4 and 0.11 ppm in the spectra of aqueous DPG and cryolysate, respectively. These chemical shifts are indicative of Cd^{2+}-DPG complex formation which is further substantiated by line broadening occurring in aqueous DPG spectra upon addition of Cd^{2+}. The magnitude of the observed Cd^{2+}-induced chemical shift is much smaller in cryolysates, even though the ratio of Cd^{2+} to DPG is approximately twice that of the aqueous sample, thus implying that other intracellular constituents are also involved in Cd^{2+} binding. This is in agreement with work done by Rabenstein et al. [9] showing that Cd^{2+} binds to glutathione and hemoglobin histidine residues in intact RBCs [6].

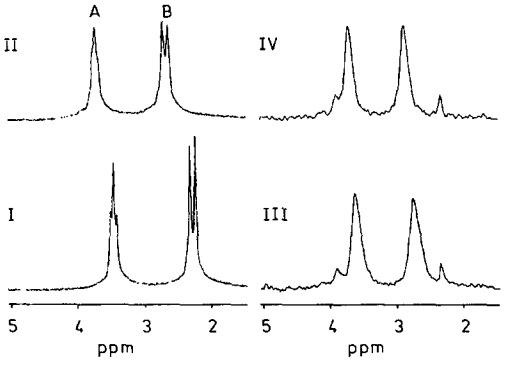

Fig.1. ^{31}P NMR spectra of DPG in aqueous solutions and in RBC cryolysates. Chemical shifts are expressed in ppm relative to 85% D_3PO_4. Spectral width, 3–5 kHz; repetition time, 1.0 s; number of scans, 1000. (I) 50 mM DPG dissolved in 0.1 M Bis-Tris buffer, pH 7.4 (in D_2O). (II) Same as (I) with 10 mM $CdCl_2$. (III) Cryolysate of fresh RBCs with an equal volume of 0.1 M Bis-Tris buffer, pH 7.4 (in D_2O). (IV) Same as (III), but with 1 mM $CdCl_2$. Assignments: (A) 3-P of DPG, (B) 2-P of DPG.

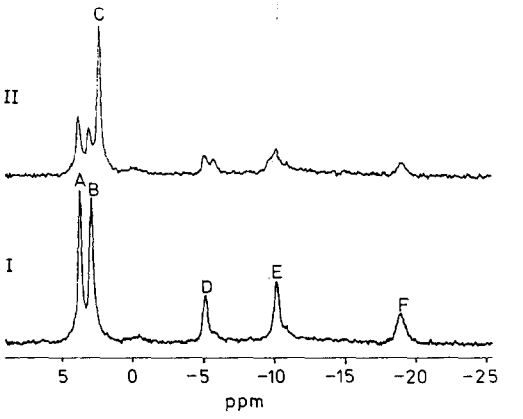

Fig.2. ^{31}P NMR spectra of intact RBCs without Cd^{2+}, fresh (I) and incubated for 7.4 h (II). Spectral width, 10 kHz; repetition time, 0.5 s; number of scans, 2000. Assignments: (A) 3-P of DPG, (B) 2-P of DPG, (C) P_i, (D,E,F) ATP.

NMR spectra of intact RBCs show no Cd^{2+}-induced chemical shift or line broadening of DPG resonances during the entire incubation period. In contrast to DPG solutions, in the cellular system these effects could be hidden due to line broaden-

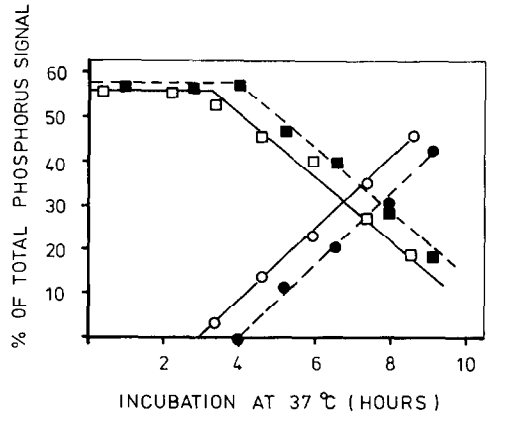

Fig.3. Breakdown of DPG in RBCs in the presence (■) or (□) absence of Cd^{2+}. The parallel increase in P_i is indicated by (○) without Cd^{2+} and (●) with Cd^{2+}.

ing caused by field inhomogeneities which could be caused by the inhomogeneous package of RBCs under non-spinning conditions, magnetic susceptibility of deoxy-Hb, and interaction of DPG with other cell constituents [13,14].

Fig.2. shows spectra of fresh RBCs and of those incubated for 7.4 h exhibiting the characteristic depletion of intracellular phosphorus metabolites [1–4]. High-resolution ^{31}P NMR makes it possible to monitor non-invasively the effect of Cd^{2+} on this depletion process. The linear decrease in DPG concentration and simultaneous increase in P_i concentration after an initial lag phase (see fig.3) are in agreement with previous reports for Cd^{2+}-free RBCs [2,6–8]. The initiation of this DPG breakdown occurs after approx. 3 h incubation in normal cells and after 4 h incubation in Cd^{2+}-treated cells, with the rate of breakdown similar in both cases. The extracellular pH, measured both prior to and after the entire incubation period, was identical for control and Cd^{2+}-treated RBCs. DPG depletion is the result of switching from DPG-formation to DPG-degradation reactions. Cd^{2+} affects the DPG-depletion process in intact RBCs. One possible mechanism which would explain this lengthening of the initial DPG lag phase is the existence of Cd^{2+}-DPG complexes.

4. CONCLUSIONS

^{31}P NMR methods provide proof for the complexation of DPG with Cd^{2+} in aqueous solution. Our results suggest that such complexation might also occur in intact RBCs. Cd^{2+} has an effect on the DPG-depletion process in intact RBCs by increasing the lag phase. The present study demonstrates the value of the non-invasive NMR method for investigation of erythrocyte cation-polyphosphate interactions.

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