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MAGNETIC RESONANCE STUDIES OF THE INTERACTION OF DIVALENT METAL CATIONS WITH 2,3-BISPHOSPHOGLYCERATE*

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Summary: The binding of Mg^{2+} to intracellular 2,3-bisphosphoglycerate in the human red blood cell is significant to the function of the cell. We have studied interactions of Mg^{2+} and Mn^{2+} with 2,3-bisphosphoglycerate by magnetic resonance spectroscopy. The results of this study reveal the presence of two independent divalent metal cation binding sites of similar affinity (K_D = 3.0 ± 0.5 mM) in the 2,3-bisphosphoglycerate molecule, one on each phosphoryl group, contrary to the assumption of one metal ion binding site made in the previous literature. Over the range of their intracellular concentrations, ATP and ADP, however, possess only one metal ion site in spite of the presence of multiple phosphoryl groups. These results are consistent with the chemistry of metal-chelation which requires the formation of 5- or 6-membered rings for the stability of chelate structures.

INTRODUCTION

P_2-glycerate, the predominant phosphorylated metabolite of the human red blood cell, plays an important role in the regulation of oxygen exchange by hemoglobin in the cell. The decrease in oxygen affinity resulting from the preferential binding of P_2-glycerate to deoxyhemoglobin serves as a fine control of hemoglobin function (1-3). Since the magnesium complex of P_2-glycerate does not interact with hemoglobin (4, 5), the binding of Mg^{2+} to P_2-glycerate affects hemoglobin function. Because the total Mg^{2+} in the red blood cell, ∿3.5 mM, is significant relative to the concentration of P_2-glycerate, an understanding of hemoglobin function in its intracellular environment requires an accurate knowledge of the interactions of Mg^{2+} with P_2-glycerate.

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Abbreviations: P_2-glycerate, 2,3-bisphosphoglycerate; bis-tris, 2,2-bis(hydroxymethyl)-2,2',2"-nitriloethanol.

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Complexation of P_2-glycerate with Mg^{2+} derives further importance from the observations that free P_2-glycerate may be a negative effector for several kinases and other enzyme systems (6-9). Thus phosphofructokinase and hexokinase, which are believed to be the two control enzymes in red cell glycolysis (10), appear to be inhibited by free intracellular levels of P_2-glycerate (6). Free P_2-glycerate, but not its Mg-complex, is a competitive inhibitor of bisphosphoglycerate mutase (9) and thus Mg^{2+} also affects the synthesis of P_2-glycerate. The intracellular availability of free Mg^{2+}, which may be an important factor affecting red cell metabolism (11), is dependent, at least in part, on the association of Mg^{2+} with P_2-glycerate. An understanding of the mechanism of glycolytic regulation in erythrocytes and the precise quantitative inter-relations between the state of hemoglobin oxygenation and red cell glycolysis, therefore, requires an accurate knowledge of the Mg^{2+}-complexing ability of P_2-glycerate. Magnetic resonance techniques provide a somewhat unique tool for studying the stoichiometry and equilibrium constant for such a system. In the course of our investigations concerning the regulation of red cell metabolism with particular reference to the role of free Mg^{2+} (12), we precisely quantitated the interactions of Mg^{2+} with P_2-glycerate, ATP and ADP by magnetic resonance methods. The results of this study revealed that contrary to the common view of a single binding site for Mg^{2+} in the P_2-glycerate molecule, there actually exist two independent binding sites of similar affinity, one on each phosphoryl group, while, at their intracellular levels, ATP and ADP have only one tight binding site. Since this finding may require re-evaluation of earlier work involving P_2-glycerate-Mg^{2+} complexes, we present a summary of our investigations in this communication.

MATERIALS AND METHODS

P_2-glycerate, as its cyclohexylammonium salt, was purchased from Calbiochem. The cyclohexylamine was removed by passage through Dowex-50 (H^+) and the free acid was neutralized with KOH. The concentration of P_2-glycerate was determined enzymatically (13).

The EPR technique was used to detect and study interactions of Mn^{2+} with phosphorylated metabolites and the displacement of Mn^{2+} by Mg^{2+}. It is known from the work of Cohn and Townsend (14) that the binding of Mn^{2+} to most molecules results in a large broadening, and hence disappearance of the EPR

resonances of Mn^{2+}, due to anisotropic environment seen by the bound metal. Thus in solutions containing Mn^{2+}, the concentration of free Mn^{2+} may be directly obtained by measuring the amplitude of the Mn^{2+} sextet EPR signal. The EPR technique is therefore useful in probing into the nature of Mn^{2+} binding sites on phosphorylated compounds. The changes in free Mn^{2+} level caused by the presence of Mg^{2+} reflect displacement of Mn^{2+} by Mg^{2+} and are used to estimate the dissociation constants for the Mg^{2+} complexes.

Phosphorus-31 NMR spectra were recorded at 40.5 MHz with a Varian XL-100-15 FT NMR spectrometer in the Fourier transform mode. All reagents were passed through columns of Chelex 100 (Biorad) and spectral grade $MgCl_2$ was used. Each spectrum was obtained as a time-average of 64 transients of free induction signal following 90° pulses. A data acquisition time of 16 sec was used to allow complete decay of the transverse magnetization. No pulse delay was used. 8 K data points were obtained over a spectral width of 250 Hz, providing a digital resolution of 0.03 Hz in the Fourier-transform NMR spectra.

RESULTS AND DISCUSSION

Electron Paramagnetic Resonance Studies- Addition of P_2-glycerate to a solution containing Mn^{2+} results in a decrease in the observed EPR signal, the effect increasing with increasing concentration of P_2-glycerate, reflecting binding of Mn^{2+}. By measuring the concentration of free Mn^{2+} at several levels of total Mn^{2+} and the organic phosphate, one can make a Scatchard plot and determine the number of Mn^{2+} binding sites in the system. Using total Mn^{2+} in the range 0.1 to 5.0 mM and total P_2-glycerate in the range 0.2 to 1.7 mM, the Scatchard plot shown in Figure 1a was obtained. All of the experimental data points fell reasonably well on a straight line. From the intercept of this straight line on the horizontal ($[Mn]_b/[P]_T$) and vertical ($[Mn]_b/[P]_T[Mn]_f$) axes, where $[P]_T$ is the total concentration of P_2-glycerate, we estimate the number of binding sites per molecule of P_2-glycerate to be two with approximately equal dissociation constants of 750 μM at pH 7.2 (T = 37°) (Table I). Similar experiments with ATP and ADP revealed only one divalent cation site in these molecules (Table I).

Addition of Mg^{2+} to a solution containing Mn^{2+} and P_2-glycerate increases the amplitude of the free Mn^{2+} EPR signal. From the apparent dissociation constant of Mn^{2+} ($K_D'^{Mn}$), measured in the presence of several levels of Mg^{2+}, and its true dissociation constant in the absence of Mg^{2+} (K_D^{Mn}), the equilibrium constant for the dissociation of Mg^{2+} from its complex with P_2-glycerate (K_D^{Mg}) is given by the following equations:

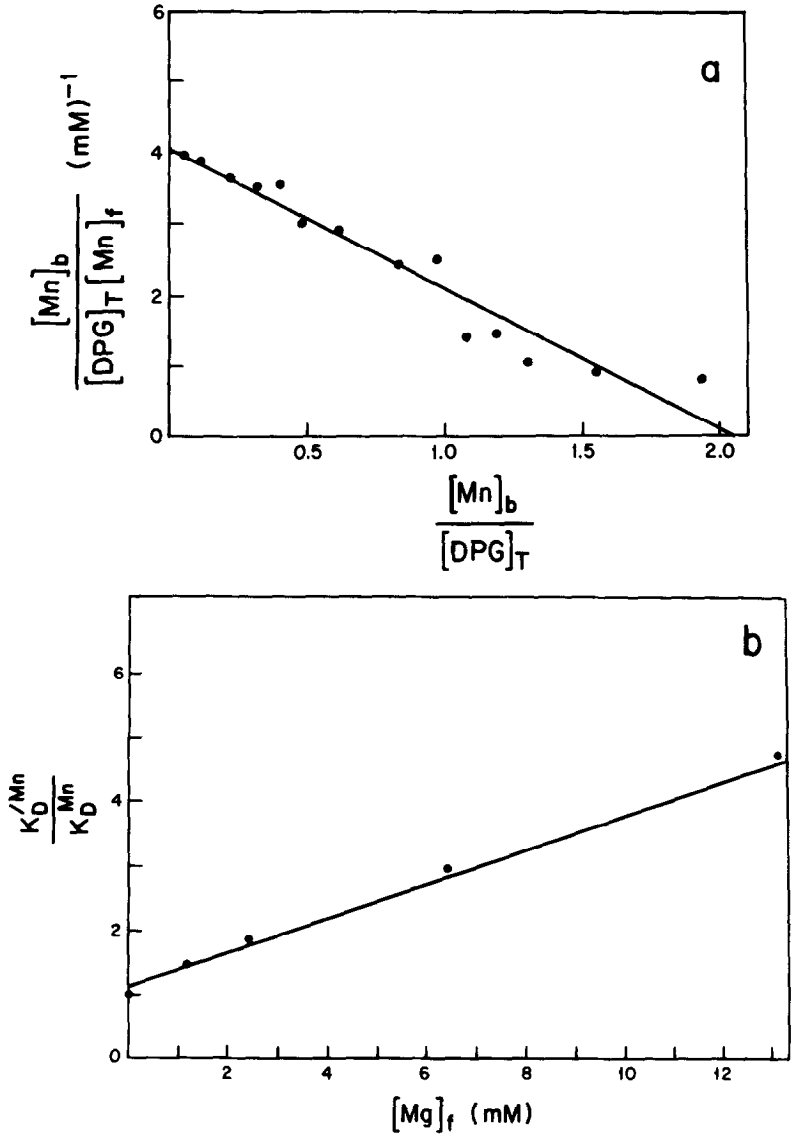

Fig. 1: (a) Scatchard plot for binding of Mn^{2+} to P_2-glycerate from EPR data. Components present were: KCl (0.15 M), tris buffer (20 mM) pH 8.1. T = 21°C. [P_2-glycerate] varied in the range of 0.2 to 1.7 mM and $[Mn]^{2+}$ was in the range 0.1 to 5 mM; (b) Graph showing displacement of P_2-glycerate-bound Mn^{2+} by Mg^{2+} based on EPR data. All solutions contained 0.50 mM P_2-glycerate, a total $[Mn^{2+}]$ level of 0.48 mM, tris buffer, pH 8.1 (20 mM) and KCl (0.15 M). T = 21°C.

$$K_D'^{Mn} = K_D^{Mn} \left(1 + \frac{[Mg]_f}{K_D^{Mg}}\right) \quad (1)$$

where $K_D'^{Mn} = \frac{[Mn]_f}{[Mn]_b} (n[P]_T - [Mn]_b)$ (2)

TABLE I

Equilibrium constants for the dissociation of Mg^{2+} and Mn^{2+} from their complexes with P_2-glycerate, ATP and ADP obtained from Scatchard plots of EPR data (μ = 0.15).

Complex	T	pH	number of sites	K_D^{Mn}	K_D^{Mg}
	°C			μM	μM
P_2-glycerate·M^{2+}	37	7.2[a]	2.00±0.31	750±120	3000±500
	21	8.1[b]	2.06±0.16	490±40	3600±300
ATP·M^{2+}	37	7.2[a]	1.09±0.13	7±1	38±6
ADP·M^{2+}	37	7.2[a]	0.95±0.09	90±10	430±50

[a] In 20 mM bis-tris buffer

[b] In 20 mM tris buffer

and $$K_D^{Mn} = \frac{[Mn]_f(n[P]_f)}{[Mn]_b} \quad (3)$$

$[P]_f$ is the concentration of free P_2-glycerate. n is the number of Mn^{2+} binding sites in the molecule. $[Mg]_f$ is obtained from equation (4):

$$[Mg]_f = [Mg]_T - (n[P]_T) + [Mn]_b \left(1 + \frac{K_D^{Mn}}{[Mn]_f}\right) \quad (4)$$

To obtain K_D^{Mg}, the $K_D'^{Mn}$ values calculated using equation (2) at several levels of Mg^{2+} are plotted against $[Mg]_f$ according to equation (1). The straight line plot thus obtained is shown in Fig. 1b. K_D^{Mg} is obtained from the slope of this plot and is given in Table I. For comparison we have also included results of a similar study with ATP and ADP. It is clear that while ATP and ADP have only one binding site for Mg^{2+} over the range of their intracellular concentrations, P_2-glycerate shows two independent binding sites invalidating previous assumptions of one binding site in this system (5, 15, 16). The observation of two independent binding sites (Hill coefficient n = 1.02 ± 0.02) of equal

affinity for divalent cations on P_2-glycerate is not entirely surprising since the molecule has two phosphoryl groups which are capable of binding metal ions independently. The two phosphoryl groups must be trans with respect to each other for the metal sites to be non-interacting. Unlike the case of ADP and ATP where the metal ion is simultaneously chelated to two and three phosphoryl groups, respectively, the absence of a chelate effect in P_2-glycerate is expected since no structures giving rise to the stable 5- or 6-membered chelate rings (17) are possible in this case. Since our results on the number of metal ion binding sites in P_2-glycerate were in conflict with the assumptions made by previous workers in the field, we sought to obtain independent evidence for the number of binding sites in this system by phosphorus-31 NMR.

Phosphorus-31 NMR Study of the Interaction of Mg^{2+} with P_2-glycerate- In the absence of proton decoupling, the phosphorus NMR spectrum of P_2-glycerate shows two multiplet resonance absorptions, one of which is a triplet arising from 3-P and the other a doublet from 2-P. The chemical shifts as well as the ^{1}H-^{31}P spin-spin coupling constants of P_2-glycerate are sensitive to complexation with Mg^{2+}. However, accurate measurements of chemical shifts require an internal phosphorus standard which does not bind Mg^{2+}. Since most phosphorylated compounds bind Mg^{2+}, and further since chemical shift measurements are known to be complicated by non-specific ionic strength effects at the high levels of Mg^{2+} needed for the present experiments (18), it was decided to use the ^{31}P-^{1}H coupling constant measured on the doublet absorption of 2-P of P_2-glycerate (Fig. 2) as an indicator of complexation. The $^{31}P(2)$-^{1}H coupling constant of P_2-glycerate changes from 9.6 to 8.7 Hz upon complexation with Mg^{2+} and is measurable with an accuracy of $\pm$0.05 Hz on modern FT spectrometers. A plot of the measured coupling constant as a function of the total Mg^{2+} available for binding is shown in Fig. 2. The concentration of P_2-glycerate was fixed at $\sim$10-fold above its dissociation constant for Mg^{2+} measured from EPR studies. It is clear that the change in the coupling constant approaches saturation at a level of $[Mg^{2+}]_T$ which is two-fold higher than the concentration of P_2-glycerate present establishing the existence of two

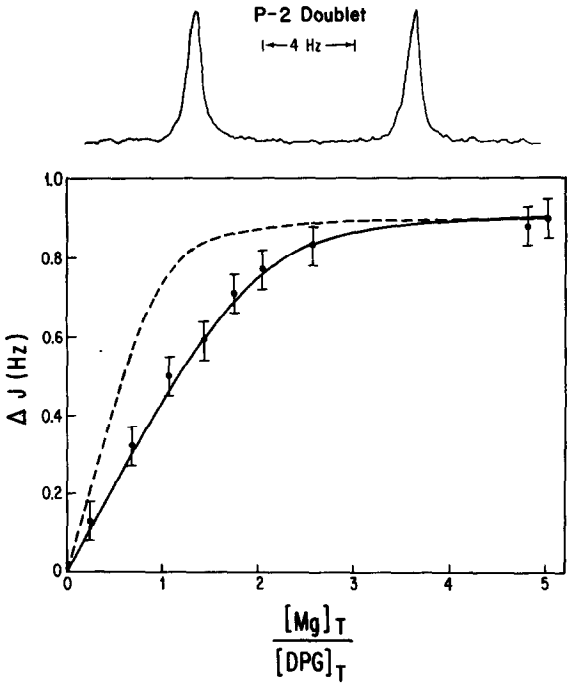

Fig. 2: (upper) The proton-coupled doublet resonance from the P-2 phosphorus atom of P_2-glycerate in the Fourier transform ^{31}P NMR spectrum of a 0.034 M solution of P_2-glycerate in 0.05 M tris buffer at pH 8.4 obtained with 64 transients of free induction signal. (lower) Graph showing variation in the ^1H-^{31}P coupling constant measured on the P-2 resonance with added Mg^{2+}. [Mg^{2+}] was varied in the range 0 to 170 mM. The solid line through the data points shows the theoretical fit obtained using the dissociation constant in Table I. The dotted line shows the theoretical curve obtained assuming one binding site with a two-fold higher affinity.

independent binding sites of equal affinities for the divalent metal in the P_2-glycerate molecule. The solid line through the data points is a theoretical fit assuming two binding sites and the dissociation constant measured from the EPR data (Table I) while the dotted line is a theoretical curve obtained assuming one binding site with a two-fold higher affinity. The one binding site assumption is clearly inconsistent with our ^{31}P-NMR and EPR data.

REFERENCES

1. Benesch, R. and Benesch, R.E. (1967) Biochem. Biophys. Res. Commun. 26, 162-167.
2. Chanutin, A. and Curnish, R.R. (1967) Arch. Biochem. Biophys. 121, 96-102.

3. Benesch, R., Benesch, R.E. and Yu, C.I. (1968) Proc. Nat. Acad. Sci., USA 59, 526-532.
4. Hamasaki, N. and Rose, Z.B. (1974) J. Biol. Chem. 249, 7896-7901.
5. Berger, H., Janig, G.R., Gerber, G., Ruckpaul, K. and Rapoport, S.M. (1973) Eur. J. Biochem. 38, 553-562.
6. Ponce, J., Roth, S. and Harkness, D.R. (1971) Biochim. Biophys. Acta 250, 63-74.
7. Tarui, S., Kono, N. and Uyeda, K. (1971) J. Biol. Chem. 247, 1138-1145.
8. Lowry, O.H. and Passonneau, J.V. (1966) J. Biol. Chem. 241, 2268-2279.
9. Rose, Z.B. (1968) J. Biol. Chem. 243, 4810-4820.
10. Rose, I.A. (1971) Exp. Eye Res. 11, 264-272.
11. Rose, I.A. (1968) Proc. Nat. Acad. Sci., USA 61, 1079-1086.
12. Gupta, R.K., Benovic, J.L. and Rose, Z.B. (1978) J. Biol. Chem., in press.
13. Rose, Z.B. and Liebowitz, J. (1970) Anal. Biochem. 35, 177-180.
14. Cohn, M. and Townsend, J. (1954) Nature 173, 1090-1092.
15. Collier, H.B. and Lam, A. (1970) Biochem. Biophys. Acta 222, 299-306.
16. Bunn, H.F., Ransil, B.J. and Chao, A. (1971) J. Biol. Chem. 246, 5273-5279.
17. Martell, A.E. and Calvin, M. "Chemistry of the Metal Chelate Compounds", Prentice-Hall, Inc., New York, 1952.
18. Son, T.D., Roux, M. and Ellenberger, M. (1975) Nucleic Acids Res. 2, 1101-1110