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A REAPPRAISAL OF ^{31}P NMR STUDIES INDICATING ENZYME COMPLEXATION IN RED BLOOD CELLS*

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Summary: ^{31}P NMR and column fractionation studies do not substantiate the existence in solution of a complex of phosphoglycerate kinase and phosphoglycerate mutase or of one involving these enzymes and glyceraldehyde-3-phosphate dehydrogenase. It is shown that the small shifts (≤3.2Hz) in the ^{31}P resonances of 2,3-bisphosphoglycerate which were interpreted to indicate the existence of such complexes (Fossel and Solomon (1977) BBA 464, 82-92) probably result from very small variations in pH (<0.1 unit). Further, no significant resonance shifts are detected in the presence of ouabain in glucose-depleted human red blood cells. An error analysis of the NMR data indicates that previously reported ouabain-induced shifts are within the noise level of the measurement and do not indicate the presence of enzyme complexes in the red cell.

INTRODUCTION

Great interest has been generated by the series of papers of Fossel and Solomon (1-4) in which they presented evidence for a link between the Na^+-K^+ pump on the outside of the red cell membrane and the 2,3-DPG[1] within the cell. Using ^{31}P-NMR, they found that ouabain added to starved red cells caused shifts of the phosphorus resonances of intracellular 2,3-DPG. Shifts in these resonances were also observed when mixtures of the purified enzymes, PGK and PGM (but neither alone), were combined in the presence of 2,3-DPG. GAPDH caused an additional shift of the 3-P resonance of 2,3-DPG. It was inferred that the enzymes formed complexes with each other, which interacted with 2,3-DPG in a unique manner.

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[1] Abbreviations: 2,3-DPG, 2,3-bisphosphoglycerate; PGM, phosphoglycerate mutase; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TES, N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid.

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We wished to extend these studies. The chemical shifts observed with the ouabain treated cells were small and it was important to be sure that they were significant. Further, the concentrations of enzymes used in this study were very low (≤10 μM) and the resonance frequency shift extrapolated to the fully bound state for the complex with the isolated enzymes was estimated to be unusually large (4), 5850 Hz at 109.3 MHz (54 ppm). Further study might provide a better understanding of the origin of such large shifts. Although the reported results were obtained when the experiments were repeated, the effects on enzymes and cells disappeared with careful control of the pH of the medium.

MATERIALS AND METHODS

Compounds of analytical purity were used throughout. PGM (rabbit muscle) and GAPDH (rabbit muscle) were from Boehringer Mannheim and PGK (yeast) was from Sigma. Sephadex G-25 coarse and G-150 superfine gels were from Pharmacia. The concentrations of enzymes were determined spectrophotometrically.

NMR experiments: Experiments were performed in 10 mM Tris or TES buffer (pH 7.4) containing 5 mM 2,3-DPG, 5 mM $MgCl_2$, 120 mM KCl and 20% D_2O. All operations prior to the NMR experiments were done at 0-4°C. On the day of the experiment salt and paramagnetic metal ions were removed from the enzymes by passing them through columns of Sephadex G-25 (medium) (20 x 1 cm) and Chelex-100 (0.5 x 0.5 cm), after which they were concentrated in collodion bags. The NMR measurements were done at 40.5 MHz and 25°C on a Varian XL-100 NMR spectrometer in the Fourier transform mode using 12 mm sample tubes. To study the effect of ouabain on human erythrocytes, cells from freshly drawn blood in sodium citrate were washed three times at 4°C in 0.9% NaCl. The cells were suspended at 20% hematocrit in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate (HEPES) buffer (pH 7.40) containing 131 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20% D_2O with or without 10 mM glucose. When present, ouabain was 1 μM. The pH was readjusted to 7.40 with 0.3 M NaOH or 0.3 M HCl as required. The cells were incubated for two hours at 37°C with shaking and the pH in each sample carefully readjusted to 7.40 before measuring the NMR spectrum.

Gel filtration to detect complexes of enzymes: Experiments were done in 10 mM bis (2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis Tris), 150 mM KCl, pH 7.2. A sample of PGM (0.7 mg) alone or mixed with PGK (0.7 mg) in 0.85 ml buffer was applied to a column of Sephadex G-150 superfine (113 x 1.1 cm). The flow rate was 1.47 ml/h and 0.65 ml fractions were collected. The enzymes were assayed spectrophotometrically according to Bergmeyer (5).

RESULTS AND DISCUSSION

NMR studies related to association of soluble enzymes: In order to increase the sensitivity of the NMR method for detecting enzyme complexation, we used enzymes at about 5 times the concentrations used in the work of Fossel and Solomon (1)

TABLE I

Enzyme mediated changes in the ^{31}P NMR spectrum of 2,3-DPG. Changes in the chemical shifts of the NMR signals from the ^{31}P nuclei of 2,3-DPG were measured in the presence of PGK, PGM and GADPH. All solutions contained Tris or TES buffer with 5 mM 2,3-DPG, 5 mM Mg^{2+} and other components described in the text. Concentrated desalted enzymes were added to the final concentrations shown in the Table. pH was measured in each sample after the NMR measurement.

enzymes[a] (mg/ml)	Change in chemical shift relative to control[b] (Hz)					
	Tris buffer			TES buffer		
	pH[c]	2-P	3-P	pH	2-P	3-P
None	7.34	0	0	7.44	0	0
PGM(1.3)	7.34	-0.3	-0.5	7.15	-10.7	-16.3
PGK(3.0)	7.13	-5.5	-8.6			
PGM(1.3) +PGK(3.0)	7.10	-6.0	-9.4	7.43	-0.7	-1.4
PGM(1.3) +PGK(3.0)	7.07	-7.3	-11.2	7.41	-1.2	-1.9
+GAPDH(0.6)				7.45	+0.6	+1.1

[a] The enzyme concentrations are ∿5-fold higher than those used in reference 1.

[b] Minus sign refers to an upfield chemical shift.

[c] Accuracy of pH measurement is estimated to be ± 0.02.

but with the same concentration of 2,3-DPG and Mg^{2+}. Therefore the chemical shifts were expected to be ∿5 times greater than those reported (1), that is 5-10 Hz. Our results are presented in Table I and show that with Tris buffer in experiments done under the same conditions as described by Fossel and Solomon (1), the presence of enzymes causes the expected larger shifts. However, the pH of the solution varied from 7.4 to 7.1. This is due to the poor buffer-capacity of 10 mM Tris at pH values below pH 7.5 (pK is 8.14). When the experiments were done with 10 mM TES buffer (pK ∿7.5), the shifts in pH were much less (Table I) and the chemical shift changes were much smaller (≤2 Hz). For example in the presence of PGM, PGK, and GAPDH the largest chemical shift was only -2.0 Hz for the 3-P; however, upon the addition of enzymes the pH (7.41) also was shifted by -0.03, compared to the control (7.44) (Table I). When the NMR spectrum of the

sample was remeasured after the pH was adjusted back to 7.45 (0.01 unit higher than the control), the chemical shift changes were reversed to values slightly higher than the control (Table I). The conclusion from these studies is that the observed chemical shift changes are attributable directly to the observed small shifts in pH. The chemical shift changes measured by Fossel and Solomon require a variation in pH of only 0.02 units according to the results in Table I. In that study no evidence was presented indicating that the pH remained constant or that the chemical shift changes were proportional to the enzyme concentrations, a necessary assumption in their extrapolation to the bound state shifts.

NMR studies of the effect of ouabain on red blood cells: The effect of ouabain on the ^{31}P chemical shifts of intracellular 2,3-DPG in cells incubated with or without glucose was also re-examined (Table II). An error analysis (by computer simulation) based on spectral signal to noise ratio and observed line widths indicated that the standard deviation associated with the determination of the position of a resonance is 0.8 Hz (see Appendix). Assuming an uncertainty of only 0.01 units in the determination of the extracellular pH and knowing the pH dependency of the ^{31}P chemical shifts of 2,3-DPG ($\sim$60 Hz per unit pH at 40.5 MHz (1.5 ppm) and pH 7.2 (Table I and (6)), there is a total standard deviation of at least 1 Hz in the positioning of the NMR signal. The standard deviation associated with the difference in resonance positions will be 1.4 Hz. Differences that Fossel and Solomon measured (1) were of the order of 1-2 Hz which are well within two standard deviations. Our own results from such experiments (Table II) showed average differences of $\leq$0.5 Hz between the starved cells with ouabain and the controls which are too small to be statistically significant. pH differences of 0.01 to 0.02 units alone would be enough to cause the observed changes in the NMR chemical shifts. Such small differences in the intracellular pH are practically impossible to determine. Therefore, no conclusions can be drawn from the NMR data regarding complexation of the red cell enzymes.

Column fractionation of a mixture of PGK and PGM: Gel filtration of a mixture of the commercially available preparations of PGK and PGM showed no indication of

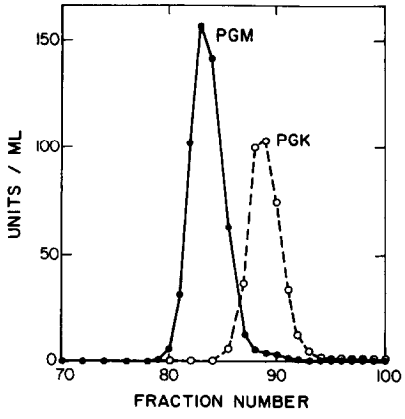

Figure 1. Gel filtration of a mixture of PGK and PGM. Experiments were done in 10 mM Bis Tris, 150 mM KCl, pH 7.2. A sample of PGM (0.7 mg) mixed with PGK (0.7 mg) in 0.85 ml buffer was applied to a column of Sephadex G-150 superfine (113 x 1.1 cm). The flow rate was 1.47 ml/h and 0.65 ml fractions were collected. The enzymes were assayed spectrophotometrically.

TABLE II

Ouabain-induced changes in the ^{31}P NMR signals of intracellular 2,3-DPG. Changes in chemical shifts of the ^{31}P NMR signals of intracellular 2,3-DPG in red blood cells were measured after 2 h starvation with and without ouabain and in red blood cells incubated with 10 mM glucose with and without ouabain. In all cases the pH was adjusted to 7.4 immediately before the ^{31}P NMR spectrum was obtained.

10 mM glucose	1 μM ouabain	chemical shift change[a] 2-P	3-P
present	absent	0	0
	present	+0.8	+0.6
		+0.2	-0.5
		-0.4	-1.0
		+0.2	+0.1
absent	absent	+0.4	-0.3
		-0.2	-1.4
		-0.0	-0.4
		+0.6	+0.7
	present	+1.5	+1.9
		+0.9	+0.8
		-0.6	-1.1
		+0.0	+0.0

[a]Several measurements were made on the same samples. Chemical shifts were measured on proton noise-decoupled ^{31}P spectra obtained with 1.6 sec acquisition time and averaging 500 transients of free induction decay signals in ∿15 min. An exponential filtering constant of 0.1 sec was used to improve spectral signal to noise ratio.

association of the two enzymes, contrary to previous reports (1). The two enzymes came out of the column only in two separate peaks (Fig. 1); each peak contained one of the enzymes at the elution volume characteristic of its molecular weight. PGM was eluted in the same volume whether applied alone or with PGK. There was no indication of a third protein band eluting prior to (i.e. heavier than) the two that contained the separate enzymes. These findings are consistent with the NMR results described and indicate no association between yeast PGK and rabbit muscle PGM. Ultracentrifugation and column fractionation studies of human red cell hemolysates indicate the absence of enzyme complexation for PGK and PGM in the red cell as well.

APPENDIX

Estimation of errors in the measurement of NMR chemical shifts. A simple estimate of a peak position in NMR spectroscopy is the location of the observed maximum in the resonance absorption. We wish to consider the error associated with such an estimate. The basic NMR signal is a Lorentzian function modulated by Gaussian noise, i.e.,

$$f(\omega) = \frac{S}{1 + (\omega-\omega_o)^2 T_2^2} + \varepsilon \qquad (1A)$$

where S is the true signal amplitude at the resonance frequency ω_o. πT_2 is the inverse of the full width of the resonance signal at half height. ε is the random noise term which is assumed to be Gaussian with a standard deviation N. In Fourier transform NMR the signal $f(\omega)$ is available only in the discrete form i.e. only a finite number of points with a constant separation are available to define $f(\omega)$. Because of this there is an uncertainty in determining the peak position even in the absence of amplitude noise. The resonance peak position will be uniformly distributed between two points, the associated standard deviation being $d/\sqrt{12}$ where d is the distance between two points.

When measurement noise ε is present, the analysis becomes more complicated. However, an estimate of error can be obtained by Monte Carlo simulation of a Lorentzian signal with noise and by calculating the standard deviation σ associated with line position from a large number of observations. Table IA shows the results of such calculations from 1000 observations of simulated discrete spectrum at each noise level. Calculations are presented for several choices of the total number of spectral points over the full width. The standard deviation N of the amplitude noise must be estimated from the data. A crude estimate of N would be peak to peak noise over a linewidth divided by ~ 5.[2] The tabulated standard deviations are expressed as the number of equally spaced points which must be multiplied by the separation between points to obtain this quantity in frequency units (Hz). In our experiments with red cells, the widths of the 2,3-DPG resonances were 12 Hz and the distance between points was 0.6 Hz. The number of points over the signal width is, therefore, ~ 20. For our observed signal to noise ratio of 20, the standard deviation σ would be 1.27 points or 0.6 x 1.27 = 0.8 Hz.

[2] See, for example, Ernst, R. R., in "Advances in Magnetic Resonance", (Waugh, J. S., Editor) Vol. 2, p. 1, 1966, Academic Press, New York.

TABLE IA

Estimation of error in the chemical shift of an NMR signal. The signal is given as a finite number of points with a constant distance between points on the frequency axis. The maximum signal is used as the position. In the Monte Carlo simulations the signal was calculated as a fixed Lorentzian function (Eq. 1A) with $S = 1$, $T_2 = 1$, and $\omega_o = 0$. The noise was simulated by a pseudo random number generator with normal deviate (Gaussian) distribution, mean = 0, and standard deviation = N. The maximum amplitude point was chosen with another pseudo random number generator with uniform distribution between two points on the frequency scale. The ω values used were in the range $\pm 10/T_2$ in all cases. The two parameters varied were the number of points over the full width (W) at half height and the signal to noise ratio (S/N). For each case the standard deviation was calculated from 1000 samples. The standard deviations given are relative to the distance (in Hz or ppm) between two points.

	Standard deviation σ (points)							
W(points)	1	2	5	10	20	50	100	
S/N								S/N
1	2.5	4.8	11.5	21	41	99	193	1
2	1.80	3.4	6.9	11.7	21	40	69	2
3	1.27	2.1	3.3	3.7	5.7	9.7	19.1	3
4	1.03	0.90	1.26	1.85	3.1	8.6	12.5	4
5	0.62	0.41	0.86	1.48	2.7	6.1	11.5	5
7	0.33	0.33	0.64	1.19	2.2	5.1	9.3	7
10	0.29	0.32	0.53	1.00	1.77	4.3	7.5	10
20	0.29	0.30	0.39	0.69	1.27	2.9	5.3	20
30	0.29	0.29	0.34	0.57	1.03	2.3	4.4	30
50	0.29	0.29	0.32	0.44	0.80	1.87	3.5	50
100	0.29	0.29	0.29	0.36	0.58	1.36	2.5	100
∞	0.29	0.29	0.29	0.29	0.29	0.29	0.29	∞

An approximate heuristically derived formula for σ (eq. 2A) gives a good fit to the data if W(S/N) >300. Interested readers may request the derivation from the authors.

$$\sigma \text{ (in points)} = \left(\frac{N/S}{16(\pi T_2)^2 d^2} + \frac{1}{12} \right)^{1/2} \qquad (2A)$$

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