Vol. 87, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 13, 1979 Pages 869-875

# THE IDENTIFICATION OF GLYCOLATE-2-P AS A CONSTITUENT OF NORMAL RED BLOOD CELLS

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Received March 2,1979

SUMMARY

Glycolate-2-P has been isolated from two samples of normal human blood at levels of 2.56 and 5.16 μM. The compound appears to be confined to the red cells. These levels of glycolate-2-P can contribute significantly to the activation of red cell bisphosphoglycerate phosphatase.

Recently we reported that the rate of glycerate-2,3-$P_2$ breakdown is accelerated in human red blood cells incubated with glycolate (1). The continued presence of glycolate was needed for this acceleration to be seen. It was proposed that the effect was due to the intracellular phosphorylation of the glycolate to glycolate-2-P, a potent activator of the glycerate-2,3-$P_2$ phosphatase of red cells (2, 3). Glycolate might be phosphorylated by pyruvate kinase which had been shown to have glycolate kinase activity (4). Subsequently the presence of a specific phosphatase for glycolate-2-P in red cells was reported (5) and this could explain the need to maintain glycolate in the medium.

The factors that normally regulate the level of glycerate-2,3-$P_2$ in the red cell are not known. Glycerate-2,3-$P_2$ phosphatase is activated by a number of anions which give widely different maximal rates (2). Although $P_i$ and $Cl^-$ together can activate, the rate of phosphatase activity attributable to these ions is too low to account for the breakdown rates observed with cells or to balance the steady state rate of glycerate-2,3-$P_2$ synthesis (6). Therefore the cell may contain more efficient effectors of the phosphatase, possibly including glycolate-2-P.

## MATERIALS AND METHODS

[$^{14}$C]Glycolate-2-P was prepared from [1-$^{14}$C]glucose which was converted to fructose bisphosphate enzymatically and then oxidized with periodate.

0006-291X/79/070869-07$01.00/0

Glycolate-2-P was purchased from General Biochemicals. Glycerate-2,3-$P_2$-[U-$^{32}$P] was prepared as reported previously (2). Dowex 1-$Cl^-$ was obtained from Bio-Rad. Enzymes used for assays were purchased from Boehringer. Bisphosphoglycerate synthase (phosphatase) was purified to apparent homogeneity from human red blood cells (7).

Assay for glycolate-2-P. Glycolate-2-P was assayed by making use of its activation of the bisphosphoglycerate phosphatase activity of red cell bisphosphoglycerate synthase. Incubations were at 25° for 10 min and contained in 0.1 ml: 10 mM glycylglycine-Na buffer, pH 7.5, 5 mM β-mercaptoethanol, 2-7 μM glycerate-2,3-$P_2$-[U-$^{32}$P]($10^7$-2 x $10^8$ cpm/μ atom $^{32}$P), 10 mM NaCl, and red cell bisphosphoglycerate synthase (phosphatase) sufficient to consume about 10% of the substrate with the added glycolate-2-P sample in the range of 1-5 nmoles. The reactions were stopped with $H_2SO_4$ and the $^{32}P_i$ released was determined by extraction of the molybdate complex (2). Neutralized fractions from the Dowex 1-$Cl^-$ columns contained 20 mM NaCl and this was considered in the maintenance of a constant salt level. More than one concentration of unknown was analyzed in each case and increments of glycolate-2-P were added to incubations with $^{14}$C-containing activator to test for possible inhibitors in the sample being analyzed.

Isolation of glycolate-2-P from normal human blood. [$^{14}$C]Glycolate-2-P was used as an internal standard of recovery.

A. Analysis of whole blood. The blood sample (150 ml) was drawn into heparin and immediately added to an ice-cooled, stirred solution containing 150 ml of 2 M perchloric acid, 5.5 mμmoles [$^{14}$C]glycolate-2-P (3.51 x $10^5$ cpm), and 20 mμmoles of glycerate-2,3-$P_2$ (1.85 x $10^5$ cpm). The solid material was removed by centrifugation at 27,000 x g for 20 minutes at 0°. The supernatant was neutralized with KOH and centrifuged at 41,000 x g at 0° for 20 minutes. The sample was applied to a column of Dowex 1-$Cl^-$ (1.8 x 30.5 cm). The column was eluted with 3 l of 0.01 N HCl which released no $^{14}$C counts, indicating that no appreciable hydrolysis of the glycolate-2-P had occurred. Elution with 0.02 N HCl released a $^{14}$C-containing peak which was combined, dried, dissolved in water and neutralized. The sample contained 1.87 x $10^5$ cpm of $^{14}$C. Red cell glycerate-P present in the $^{14}$C sample was removed enzymatically with phosphoglycerate mutase, enolase, pyruvate kinase, and lactate dehydrogenase. The sample contained only 0.01 μmole of glycerate-2-P and 0.02 μmole of glycerate-3-P indicating that the bulk of these compounds had been separated from the $^{14}$C peak on the column. Separate control experiments confirmed that glycerate-2-P and glycolate-2-P can be separated under the experimental conditions. The $^{14}$C-containing sample was diluted to 20 ml and applied to a second column of Dowex 1-$Cl^-$ (1 x 20 cm). The column was washed with water (50 ml) and 0.01 N HCl (200 ml). A single $^{14}$C-containing peak was eluted with 0.02 N HCl (3.3 ml fractions) which contained 6 x $10^4$ cpm or 17% of the $^{14}$C added to the blood.

B. Separate analyses of red cells and serum plus buffy coat. A fresh sample of heparinized blood was centrifuged at 1000 x g for 10 min at 25°. The serum and buffy coat were removed and combined. Six ml of red cells or the serum fraction were added quickly to 6 ml cold 2 M $HClO_4$ containing 0.8 mμmole $^{14}$C-glycolate-2-P (5.14 x $10^4$ counts) while mixing rapidly. The procedure was continued as for whole blood with appropriate reductions in the column sizes. Each fraction was purified on only one Dowex 1-$Cl^-$ column.

## RESULTS AND DISCUSSION

### Determination of the Glycolate-2-P Level in Human Red Blood Cells.

To analyze the whole blood sample, individual fractions from the second Dowex 1-$Cl^-$ column were counted and assayed for glycolate-2-P. The low $K_a$ of 8 μM

(8) is favorable for obtaining appreciable rates with glycolate-2-P levels as low as 0.01 μM. The results in Table I show that the glycolate-2-P content of the cells as determined enzymatically parallels the $^{14}C$ counts. Increments of glycolate-2-P added as internal standards in the enzymatic assays give the expected increases in rate indicating the absence of inhibitory compounds in the fractions. As calculated from the total counts added to the initial extract, the total glycolate-2-P in the blood sample was 126 mμmoles. Assuming that the compound is contained entirely in the red cells and allowing for 0.7 as the water content of the cells, the concentration of glycolate-2-P in the red cells was 2.56 μM.

In order to be sure that the glycolate-2-P is indeed confined to the red cells, a sample of blood was divided into a red cell fraction and one containing serum plus buffy coat (with some contaminating red cells). The two fractions were analyzed in the same way as the whole blood. The glycolate-2-P was found only in the red cells and the concentration was 5.16 μM.

Regulation of the Glycerate-2,3-$P_2$ Level in the Red Cell. In the steady state the rate of synthesis of glycerate-2,3-$P_2$ must equal the rate of breakdown. The enzyme bisphosphoglycerate synthase (glycerate-1,3-$P_2$ → glycerate-2,3-$P_2$) is believed to be responsible for both the synthesis and hydrolysis of glycerate-2,3-$P_2$ in the human red blood cell (8, 9). The rate of synthesis of glycerate-2,3-$P_2$ predicted from the kinetic properties of the isolated enzyme is 0.17 μmole/hr/ml of packed cells or about 7% of the glycolytic flux.

The phosphatase rate will depend on the sum of the effects of the various activators present in the cell. $P_i$ together with $Cl^-$ stimulate at their physiological levels (2) but can account for only a small part of the hydrolytic activity. A number of other red cell metabolites have little effect on the phosphatase rate when tested near their intracellular levels in the presence of $P_i$ (Table II). Also ineffective were citrate (0.1-0.5 mM), lactate (0.5-2 mM), pyruvate (1.25-7.5 mM) and fructose-1,6-$P_2$ (2).

TABLE I

Correlation between glycolate-2-P and $^{14}C$ content of fractions from Dowex 1-$Cl^-$ column

| Fraction # | cpm / ml of fraction | [P-glycolate] µM | cpm / [P-glycolate] |
|---|---|---|---|
| 25 | 27 | 0 | |
| 28 | 37 | 0.020 | 1850 |
| 32 | 201 | 0.090 | 2233 |
| 36 | 919 | 0.330 | 2785 |
| 43 | 294 | 0.105 | 2800 |
| 47 | 109 | 0.048 | 2271 |
| 51 | 56 | 0.022 | 2545 |

TABLE II

Effects of physiological levels of $P_i$ and some other metabolites on glycerate-2,3-$P_2$ phosphatase activity

| Additions (concentration) | Relative Rate pH 7.0 | pH 7.5 |
|---|---|---|
| None | .20 | |
| ATP (1 mM) | .26 | .25 |
| Pi (1 mM) | 1.00 | 1.00 |
| $MgCl_2$ (2.5 mM) | .18 | .18 |
| ATP (1 mM), $MgCl_2$ (2.5 mM) | .23 | .20 |
| Pi (1 mM), ATP (1 mM) | .84 | .87 |
| Pi (1 mM), ATP (1 mM), $MgCl_2$ (2.5 mM) | .82 | .85 |
| Pi (1 mM), PEP (10 µM) | | .81 |
| Pi (1 mM), PEP (50 µM) | | .70 |
| Pi (1 mM), glycerate-3-P (50 µM) | | .01 |
| Pi (1 mM), $NaHCO_3$ (20 mM) | 1.02 | .78 |
| Pi (1 mM), $NaHCO_3$ (50 mM) | .86 | .57 |
| Pi (1 mM), glycolate-2-P (4 µM) | | 4.15 |

Incubations were for 10 min at 37° and contained in 0.2 ml: 10 mM TES-Na buffer, 5 mM β-mercaptoethanol, 60 mM KCl, .04 mg bovine serum albumin, 1 µM glycerate-2,3-[U-$^{32}P$], and 4 x $10^{-6}$ µmole phosphatase subunits.

TES = N-tris[hydroxymethyl]methyl-2-aminoethane sulfonate-Na

The $K_m$ of glycerate-2,3-$P_2$ determined in kinetic studies is in the micromolar range (2). Experiments with glycerate-2,3-$P_2$ at 0.25 and 0.5 mM

indicated no change in the $K_a$ for $P_i$ or the specific activity at saturation. Maximal rates of the synthase and phosphatase reactions of purified enzyme and the levels of these activities in red cells are summarized in Table III.

Calculation of the Phosphatase Rate in the Red Cell. The rate of the phosphatase reaction, v', will not depend on the glycerate-2,3-$P_2$ concentration, which is always saturating in the cell, but on the levels and respective maximal rates of activators, and the total phosphoglycerates (PGA). These considerations lead to equation 1:

$$v' = \sum_n \left( \frac{V_{C_n}}{1 + \frac{K_{a(C_n)}}{[C_n]} \left(1 + \frac{[PGA]}{K_{i(PGA)}}\right)} \right) f_n \qquad (1)$$

where C is any activator, $V_C$ is the maximal velocity for that activator, and $K_a$ is the concentration of activator that gives half of the maximal response. The fraction (f) of the total enzyme ($E_t$) functioning at a particular rate will be related to the ratio of $K_a$ and the concentration of the activator.

$$E_t = \sum_n \left( \frac{[C_n]}{K_{a(C_n)}} \right) f_n \qquad (2)$$

To calculate the phosphatase rate expected in the cell considering only activation by $P_i$ plus $Cl^-$ and glycolate-2-P, we can solve equation 2 using for $P_i$:$K_a$ = 0.7 mM and 1 mM as the average intracellular concentration and for glycolate-2-P:$K_a$ = 8 μM and 4 μM as the concentration. This indicates that 74% of the enzyme will function with $P_i$ and $Cl^-$ and 26% will use glycolate-2-P. The net rate for the phosphatase as calculated from equation 1 (using 90 μM as the concentration of the combined glycerate-P's) is 0.083 μmole/hr/ml packed cells of which 0.015 μmole is via $P_i$ and $Cl^-$ activation and 0.068 μmole via glycolate-2-P which is half of the calculated rate of synthesis.

Conclusion: Low levels of glycolate-2-P have been demonstrated in two samples of normal human blood. The levels are sufficient to appreciably enhance the phosphatase activity of red cell bisphosphoglycerate synthase. The phospha-

TABLE III

Maximal rates of reactions catalyzed by bisphosphoglycerate synthase at 37°, pH 7.5

| Reaction | $k_{cat}$ $s^{-1}$ | $V_{max}$ in red cell µmoles/hr/ml cells |
|---|---|---|
| Synthase (glycerate-2,3-$P_2$ → glycerate-P + $P_i$ | 20[a] | 240 |
| Phosphatase with $P_i$ + 0.1 M KCl | .11 | 1.3 |
| Phosphatase with glycolate-2-P | 4 | 48 |

[a] Determined as 10 $s^{-1}$ at 25° (4).

tase activity of phosphoglycerate mutase would not contribute appreciably because of its lesser amount in the cell and higher $K_a$ for glycolate-2-P. Triose phosphate isomerase is inhibited by µmolar glycolate-2-P (10) but the enzyme is present in excess in the red cell. It was shown earlier that red cells incubated with glycolate break down glycerate-2,3-$P_2$ at an accelerated rate and it was inferred that this was due to the intracellular formation of glycolate-2-P (1). The effect of glycolate was diminished greatly in a sample of pyruvate kinase deficient cells which was in agreement with the hypothesis that the effect was due to glycolate-2-P which had been phosphorylated by pyruvate kinase (1). The present study indicates that pyruvate kinase plus glycolate-2-P phosphatase may function to maintain a low steady state level of glycolate-2-P in the red cell. It is clear that our knowledge is still incomplete regarding the factors involved in the maintenance of the steady state levels of glycerate-2,3-$P_2$ and that are responsible for the changes that are observed when cellular conditions are varied.

ACKNOWLEDGEMENTS

This work was supported by United States Public Health Service Grants GM-19875, CA-06927, RR-05539 and also by an appropriation from the Commonwealth of Pennsylvania.

REFERENCES

1. Rose, Z. B. (1976) Biochem. Biophys. Res. Commun. 73, 1011-1017; ibid (1977) 75, 217.
2. Rose, Z. B., and Liebowitz, J. (1970) J. Biol. Chem. 245, 3232-3241.
3. Rose, Z. B., and Dube, S. (1976) J. Biol. Chem. 251, 4817-4822.
4. Kayne, F. J. (1974) Biochem. Biophys. Res. Commun. 59, 8-13.
5. Badwey, J. A. (1977) J. Biol. Chem. 252, 2441-2443.
6. Momsen, G., and Vestergaard-Bogind, B. (1978) Arch. Biochem. Biophys. 190, 67-84.
7. Rose, Z. B., and Dube, S. (1976) Arch. Biochem. Biophys. 177, 284-292.
8. Rose, Z. B., and Whalen, R. G. (1973) J. Biol. Chem. 248, 1513-1519.
9. Rosa, R., Gaillardon, J., and Rosa, J. (1973) Biochem. Biophys. Res. Commun. 51, 536-542.
10. Hartman, F. C., La Muraglia, G. M., Tomozawa, Y., and Wolfenden, R. (1975) Biochemistry 14, 5274-5279.