INHIBITORY EFFECTS OF 2,3-DPG ON ENZYMES OF PURINE NUCLEOTIDE METABOLISM

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The effect of 2,3-diphosphoglycerate (2,3-DPG) upon three partially purified enzymes from human erythrocytes, 5-phosphoribosyl-1-pyrophosphate synthetase (PRibPP synthetase, E.C.2.7.6.1), IMP:pyrophosphate phosphoribosyltransferase (E.C.2.4.2.8 HPRibTase) and AMP:pyrophosphate phosphoribosyltransferase (E.C.2.4.2.7 APRibTase) has been studied kinetically. PRibPP synthetase was not greatly inhibited at physiologic concentrations of 2,3-DPG at optimal phosphate concentration, but it was inhibited appreciably by 2,3-DPG at physiologic phosphate levels. Both HPRibTase and APRibTase were inhibited by 2,3-DPG at its normal intracellular concentration. 2,3-DPG is a competitive inhibitor with respect to the substrates, ribose-5-phosphate and 5-phosphoribosyl-1-pyrophosphate. The Ki values for these inhibitions are given and the possible physiologic significance of these findings are discussed.

INTRODUCTION

2,3-diphosphoglyceric acid (2,3-DPG) has been known as one of the most abundant organic phosphate compounds in the mammalian erythrocyte since 1925 (1). It was not until 1967 (2,3) that the physiologic significance of this compound was clarified. Besides its regulatory function on the oxygenation of hemoglobin, 2,3-DPG was also found to effect the activity of several erythrocyte enzymes. Among them are hexokinase (4,5), phosphofructokinase (6,7), 6-phosphogluconate dehydrogenase (8), glucosephosphate isomerase (9), phosphoglycerate kinase and pyruvate kinase (10), transaldolase and transketolase (11), adenylate deaminase (12) and 5-phosphoribosyl-1-pyrophosphate synthetase (PRibPP synthetase) (13). The compound regulates its own rate of synthesis by product inhibition of diphosphoglycerate mutase, the enzyme responsible for its synthesis (14).

We are reporting here the pronounced inhibitory effect of 2,3-DPG upon two other partially purified purine metabolic enzymes, IMP pyrophosphate phosphoribosyltransferase (HPRibTase) and AMP pyrophosphate phosphoribosyl-

transferase (APRibTase) from human erythrocytes. They were both found to be inhibited at the intracellular level of 2,3-DPG, in contradiction to previous reports that 2,3-DPG has no effect on the crude preparations of these two enzymes (15). Since no de novo biosynthesis occurs in erythrocytes, the constant level of endogenous PRibPP is maintained by the balance between its formation and its utilization through the transferases. Our finding that 2,3-DPG is not only a potent inhibitor of PRibPP synthetase, but also an effector for the action of HPRibTase and APRibTase, further signify the important regulatory role of 2,3-DPG in erythrocyte metabolism.

MATERIALS AND METHODS

Pentahexylammonium 2,3-DPG and 5-phosphoribose, and sodium 5-phosphoribosyl-1-pyrophosphate were obtained from Sigma Chemical Co.

Determination of protein concentration: Hemoglobin was determined as methemoglobin cyanide (16). Total protein was determined by the method of Lowry et al. (17).

Enzyme assays: The assays of APRibTase and HPRibTase activity were carried out as described previously (18,19). PRibPP synthetase activity was determined by a modification of the method of Hershko et al. (13). The assay mixture contained 70 mM Tris buffer pH 7.4, 0.7 mM ethylene diaminetetraacetate, 1.7 mM mercaptoethanol, 7 mM magnesium ion, 33 mM potassium phosphate at pH 7.4, 0.17 mM ribose-5-phosphate, 0.17 mM ^{14}C-ATP (specific activity = 0.8 Ci/mole) and partially purified enzyme in a final volume of 150 µl. Each sample was incubated in a 37°C shaker bath for a definite period of time. The reaction was terminated by immersion in an ethanol-solid CO_2 bath, followed by addition of 25 µl of 40% TCA. A 25 µl aliquot of the deproteinized reaction mixtures was spotted on Whatman paper No. 1, and chromatography was carried out in a mixture of butanol, acetic acid and water 2:1:1 (v:v:v:) for 18 hours after addition of unlabeled markers. AMP spots were cut out and immersed in scintillation fluid [0.01%, 1,4-bis-2-(4-methyl-5-phenyloxazoly)] benzene and 0.5% 2,5-

diphenyloxazole in toluene) and radioactivity determined. Blanks had everything except ribose-5-phosphate in the reaction mixture.

Enzyme purification: APRibTase was partially purified by the method described previously (18). The preparation used in these studies had a specific activity of 0.2 IU/mg protein (ca 850 fold purified).

HPPibTase was partially purified by previously described procedures (19). The preparation used in these studies had a specific activity of 0.5 IU/mg protein (ca 350 fold purified).

Purification of PRibPP synthetase was carried out by a modification of the method of Fox and Kelley (20). Human erythrocyte lysate was freed of hemoglobin by absorption on DEAE sephadex and elution with 0.5 M KCl. The enzyme was precipitated from the hemoglobin-free solution in the 33-52% ammonium sulfate cut. After chromatography on sepharose 6B and then DEAE sephadex the PRibPP synthetase had reached about 500 fold purification, and had a specific activity of 0.4 IU/mg protein.

RESULTS

Studies on the effect of 2,3-DPG on PRibPP synthetase--Fig. 1 shows the competitive inhibition of 2,3-DPG on PRibPP synthetase with respect to ribose-5-phosphate. The Ki value obtained from a plot of 1/V vs [I] as shown in Fig. 1 (21) is 8.75 mM for 2,3-DPG. The insert shows a double reciprocal plot of initial velocity studies with varying amounts of ribose-5-phosphate and a fixed amount of ATP. It gives a Km value of 35 μM for ribose-5-phosphate which is very close to the value reported by Fox and Kelley with their purified enzyme.

2,3-DPG inhibition of APRibTase--Competitive inhibition of partially purified APRibTase by 2,3-DPG is shown in Fig. 2. The Ki value is 0.22 mM.

Inhibition studies on HPRibTase--Competitive inhibition of HPRibTase by 2,3-DPG is shown in Fig. 3. The Ki value for this inhibition is 0.1 mM. The smaller Ki value obtained with HPRibTase indicates that HPRibTase is the most sensitive to 2,3-DPG inhibition of the three enzymes studied.

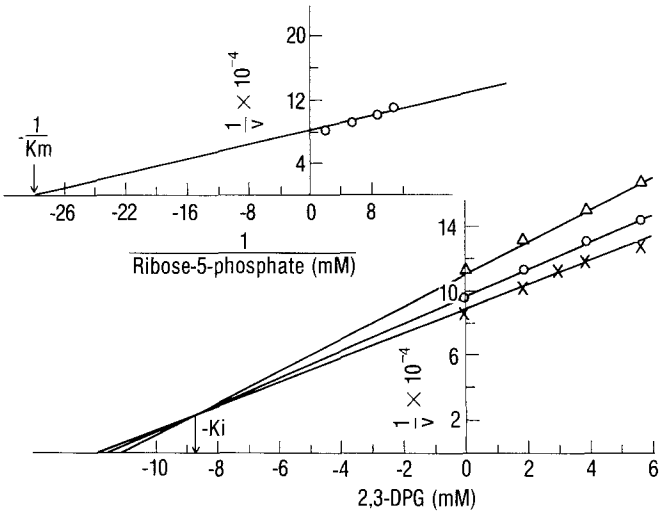

Fig. 1 Kinetic studies of the inhibition of partially purified erythrocyte PRibPP synthetase by 2,3-DPG. The reciprocal of the initial velocity is plotted against the concentration of 2,3-DPG present in the reaction mixture at three different ribose-5-phosphate concentrations; 0.0925 mM (Δ), 0.122 mM (O) and 0.185 mM (X). The reaction mixture contained 5 μg PRibPP synthetase and varying amounts of 2,3-DPG. Insert shows a double reciprocal plot of initial velocity studies with variable ribose-5-phosphate concentration and fixed Mg^{++} ATP concentration. The reaction velocity (V) is expressed as mμ moles of AMP formed/min.

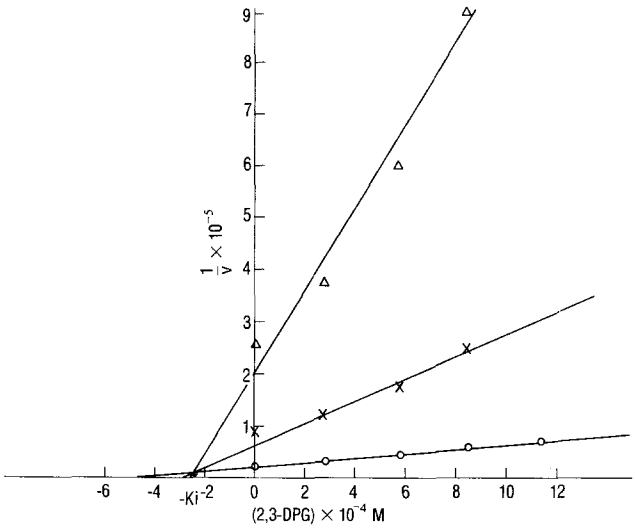

Fig. 2 Kinetic studies of the inhibition of partially purified erythrocyte APRibTase by 2,3-DPG. The reciprocal of the initial velocity is plotted against the concentration of 2,3-DPG present in the reaction mixture at three different PRibPP concentrations; 10.4 μM (Δ), 20.8 μM (X) and 83 μM (O). The reaction mixture contained 0.2 μg of enzyme, and varying amounts of 2,3-DPG. The reaction velocity (V) is expressed as mμ moles of AMP formed/min.

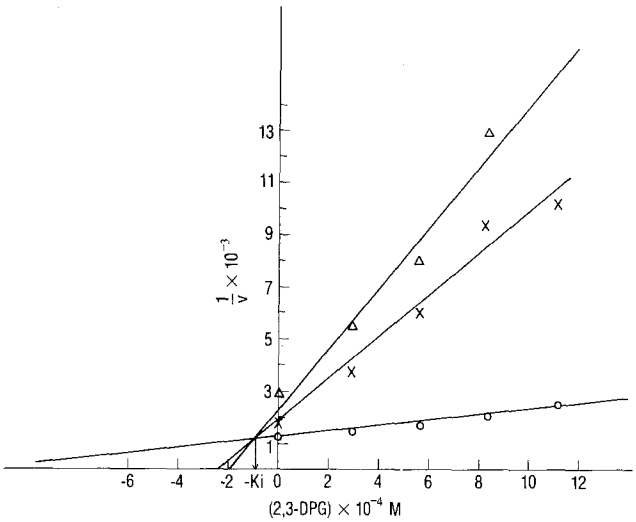

Fig. 3 Kinetic studies of the inhibition of partially purified erythrocyte HPRibTase by 2,3-DPG. The reciprocal of the initial velocity is plotted against the concentration of 2,3-DPG present in the reaction mixture at three different PRibPP concentrations; 10.4 μM (Δ), 20.8 μM (X) and 83 μM (O). The reaction mixture contained 0.12 μg of enzyme and varying amounts of 2,3-DPG. The reaction velocity (V) is expressed as mμ moles of IMP formed/min.

The sodium salt and cyclohexylammonium salt of 2,3-DPG produced the same inhibitory effects.

DISCUSSION

The data presented here indicate that 2,3-DPG has a strong inhibitory effect on APRibTase and HPRibTase, critical enzymes of purine metabolism. Furthermore, 2,3-DPG is an effector of both enzymes at normal intracellular concentrations.

We have obtained a Km value of 35 μM (Fig. 1) for ribose-5-phosphate with erythrocyte PRibPP synthetase which is consistent with the reported value for this enzyme (20). The small difference in the 2,3-DPG inhibition constant obtained under our experimental conditions, is not unexpected since we used higher phosphate concentration in our PRibPP synthetase assay, and it has been shown by Hershko et al. (13) that the inhibition by 2,3-DPG of PRibPP synthetase is partially relieved by higher concentrations of inorganic phosphate. The endogenous phosphate concentration is about 1 mM in normal erythrocytes (22).

Extrapolation to this low phosphate level enables us to conclude that even though the Ki for PRibPP synthetase is greater than the intracellular concentration of 2,3-DPG at high phosphate concentration, the inhibition might play an important regulatory role on the slow but vital *in vivo* synthesis of PRibPP. It once again emphasizes that experimental results with purified enzymes, assayed under optimal reaction condition, do not necessarily correlate directly with the actual enzyme behavior *in vivo*.

Although 2,3-DPG has been shown to have an effect on many erythrocyte enzymes, the actual regulatory mechanism still remains a subject of speculation. The accumulated data in recent years have pointed out that the formation of 2,3-DPG in the mammalian erythrocytes is closely connected with the operation of the glycolytic system, which is the only energy yielding pathway present in these cells (22,23,24). Under certain conditions of impaired glycolysis, such as in pyruvate kinase deficiency, an elevated level of 2,3-DPG in cells is accompanied by a decline of the ATP level (25). Therefore it has been suggested that the role of 2,3-DPG is to regulate the ATP level in cells (26).

The stimulation of PRibPP synthesis by glucose, fructose and mannose and the inhibition by iodoacetate and dinitrophenol have been considered as indications of the importance of glycolytic intermediates in regulating ribose-5-phosphate availability for PRibPP synthesis (27). The formation of PRibPP is of central importance because of its involvement both in the purine phosphoribosyltransferase reactions and in the *de novo* biosynthesis of purines. At the same time, the availability of ribose-5-phosphate is an essential factor controlling the rate of PRibPP synthesis in mammalian cells. The regulatory interaction among the erythrocyte metabolic pathways is further implicated by the observation that the pathogenesis of excess uric acid production in some patients with primary gout, Lesch-Nyhan syndrome and Type I glycogen storage disease is accompanied with an increased intracellular level of PRibPP (28,29,30).

The erythrocyte 2,3-DPG concentration is 5.7 mM (22). The amount available for effecting enzyme activity fluctuates, depending upon the hemoglobin

oxygenation state. The fact that 2,3-DPG is not only a regulator for the glycolytic pathway, an effector for the PRibPP synthesis but also a potent inhibitor for the purine phosphoribosyltransferases is a strong indication of its key role as a regulator for maintaining erythrocyte metabolic balance.

The presence of inosine or inosine plus adenine during erythrocyte storage increases the in vitro synthesis of 2,3-DPG and thus improves erythrocyte survival (31,33). The vitalizing effect of the added nucleoside is due to its supplying of large amounts of ribosephosphate which is able to enter the metabolic pool beyond the storage lesion and generate high energy bonds and free hypoxanthine. The strong inhibitory effect of 2,3-DPG upon HPRibTase, the enzyme responsible for synthesizing nucleotides from hypoxanthine, and the rapid disappearance of this compound during storage, again reveals the important regulatory role of 2,3-DPG. Addition of inosine restores 2,3-DPG level as well as the vitality of the cells.

We have previously reported that as erythrocytes age, the endogenous PRibPP level increases (34). Others have observed a decreased level of 2,3-DPG in aging erythrocytes (35,36.37). In view of the strong regulatory effect of 2,3-DPG on the transferases, it is possible that the abnormal levels of 2,3-DPG in some primary gout patients, might be an important factor contributing to their pathogenic excess uric acid production.

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