Biochimica et Biophysica Acta, 601 (1980) 372—379
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BBA 78925

GUANOSINE TRIPHOSPHATASE ACTIVITY IN HUMAN ERYTHROCYTE MEMBRANES

ERNEST BEUTLER and WANDA KUHL

Department of Hematology, City of Hope Medical Center, 1500 E. Duarte Road, Duarte, CA 91010 and Department of Clinical Research, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037 (U.S.A.)

(Received January 2nd, 1980)

Key words: ATPase; GTPase; (Erythrocyte membrane)

Summary

Human red cell membranes have the capacity to hydrolyze enzymatically GTP to GDP. The reaction requires magnesium, is not appreciably affected by sodium, potassium or calcium, and is not inhibited by ouabain. Kinetic analysis suggests that there are two separate enzymes in membranes which cleave GTP, a 'high K_m' GTPase and a 'low K_m' GTPase. Both enzymes are also ATPases, with an approximately equal affinity for GTP and ATP. GTPase activity did not extract from the membrane with spectrin and was not inactivated by anti-spectrin antibody. Activity was partially destroyed by 0.5% Triton X-100. It seems probable that the low K_m GTPase is the sodium- and potassium-independent ATPase of red cell membranes. The identity of the high K_m enzyme is not clear.

Introduction

Guanosine triphosphate (GTP) is a normal constituent of human erythrocytes [1,2], and is actively turned over in red cells [3]. Addition of guanosine to blood preservatives has been found to increase the viability of stored red cells [4]. Yet, the role of guanosine nucleotides in red cell metabolism remains totally undefined.

We have investigated the enzymatic hydrolysis of GTP in human erythrocyte membranes because this might ultimately shed light upon its role in red cell metabolism. The GTP-hydrolyzing activity of membranes appears to be a function of enzymes which are also ATPases.

Materials and Methods

Human blood was obtained from normal donors, defibrinated, and filtered through microcrystalline cellulose-α-cellulose to remove leukocytes and platelets, as previously described [5]. The erythrocytes were washed twice in 0.154 M NaCl. Red cell membranes were prepared by lysing the washed erythrocytes in 30—50 vols. of ice-cold 10 mM Tris-HCl buffer, pH 7.4 (25°C), and washing three or four times in the same buffer to obtain white ghosts.

Unless otherwise indicated, GTPase activity was measured at 37°C in a 250 μl system comprised of the following: Tris-HCl, pH 8.0, 100 mM; EDTA, 0.5 mM; $MgCl_2$, 10 mM; [^{32}P]GTP as specified; and stromal preparation, 0.1 to 0.4 mg. The reaction was stopped after 30 min by the addition of 28 μl of 50% trichloroacetic acid. The tubes were centrifuged in the cold and inorganic phosphate extracted from the supernatant into butyl acetate or isobutanol/benzene (1 : 1) as a molybdate complex as described by Rose and Liebowitz [6]. ATPase was assayed using the same system but substituting [^{32}P]ATP for GTP.

Assays for guanosine diphosphate (GDP) were carried out using the pyruvate kinase reaction as described for assay of ADP [7]. Guanosine monophosphate was estimated using the system described for AMP determination [7], but substituting guanylate kinase for adenylate kinase. 1 unit of all enzyme activities represents the quantity required to convert 1 μmol of substrate per min.

Guanosine triphosphate and ATP labeled with ^{32}P in the gamma position were synthesized enzymatically as previously described [8]. [^{3}H]GTP labeled in the 8-position was obtained from Schwarz/Mann.

Radioactivity of ^{32}P was determined in a liquid scintillation counter utilizing the Cerenkov effect [9]. Tritium was counted in Handifluor (Mallinckrodt). Chromatographic separation of inorganic phosphate and nucleotides was achieved by chromatography on 1.5 × 23 cm Dowex-1 columns utilizing a 2 l linear 0—5 M formic acid/ammonium formate (4 : 1) gradient modified slightly from that of Bartlett [10]. Outward GSSG transport by red cell membranes was measured as previously described [11] using ^{3}H instead of ^{35}S-labeled GSH.

Results

Effect of metals and of ouabain on GTPase activity

Red cell membranes were dialyzed overnight against 10 mM Tris-HCl, pH 7.4. They were then incubated in a system buffered with 100 mM Tris-HCl, pH 8.0 (25°C) with or without added Mg^{2+}, Na^{+}, K^{+}, Li^{+}, or Ca^{2+} and ouabain as indicated in Table I. The formation of P_i from GTP is enhanced by Mg^{2+} but is scarcely affected, if at all, by the presence of Na^{+}, K^{+} or Ca^{2+}. The activity is insensitive to the inhibitory effect of ouabain. Similar results were obtained in another experiment utilizing membranes in which GDP formation was measured enzymatically instead of estimating $^{32}P_i$ cleavage from [^{32}P]GTP.

The effect of substrate concentration on GTPase activity

The effect of GTP concentration on red cell membrane GTPase activity

TABLE I

THE EFFECT OF VARIOUS METALS AND OUABAIN ON RED BLOOD CELL MEMBRANE GTPase ACTIVITY

Enzyme activity was measured by the release of $^{32}P_i$ from [^{32}P]GTP in a system containing 0.1 M Tris, pH 8, 0.5 mM EDTA, 0.3—0.35 mg stromal protein, and additions as indicated. $^{32}P_i$ was extracted as the phosphomolybdate complex into isobutanol/benzene as described in the text.

Additions	0.1 mM GTP	2.0 mM GTP
None	0.05	0.26
Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.35	1.62
Na^+ (0.05 M), Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.43	1.66
K^+ (0.05 M), Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.40	1.65
Na^+ and K^+ (0.05 M), Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.43	1.72
Ca^{2+} ($1 \cdot 10^{-3}$ M), Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.36	1.74
Li^+ ($1 \cdot 10^{-3}$ M), Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.32	1.60
Ouabain ($1 \cdot 10^{-4}$ M), Mg^{2+} ($1 \cdot 10^{-2}$ M)	0.35	1.44
Na^+, K^+, Mg^{2+} and ouabain	0.45	1.54

consistently showed a two-component Lineweaver-Burk plot, as illustrated in Fig. 1. A GTPase activity requiring high GTP concentrations manifested a K_m value of 6.0 mM. In contrast, at low substrate concentrations, GTPase activity with a K_m of 0.4 mM was observed.

Identification of the reaction products

The products of GTP cleavage were investigated by using as substrate a mixture of tritiated GTP (Schwarz/Mann) and [γ-^{32}P]GTP. After incubation of red cell stroma with 0.1 and 2.0 mM of the doubly labeled GTP for 15 and 30 min, the reaction was terminated by the addition of 1 vol. of 10% trichloroacetic acid. The diethyl ether-extracted supernatant was chromatographed on Dowex-1 formate (Fig. 2). ^{32}P activity was found in the inorganic phosphate peak, eluting at approx. 1 M formate and in GTP, eluting at 3.7

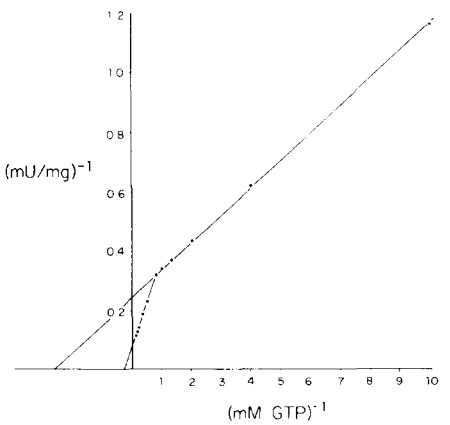

Fig. 1. Lineweaver-Burk plot for red blood cell membrane GTPase activity assayed in 0.1 M Tris, pH 8.0, 0.5 mM EDTA, 0.01 M $MgCl_2$, 0.3—0.35 mg stromal protein and GTP ranging from 0.1 to 2.0 mM. $^{32}P_i$ released was determined after extraction as the phosphomolybdate complex as described in the text.

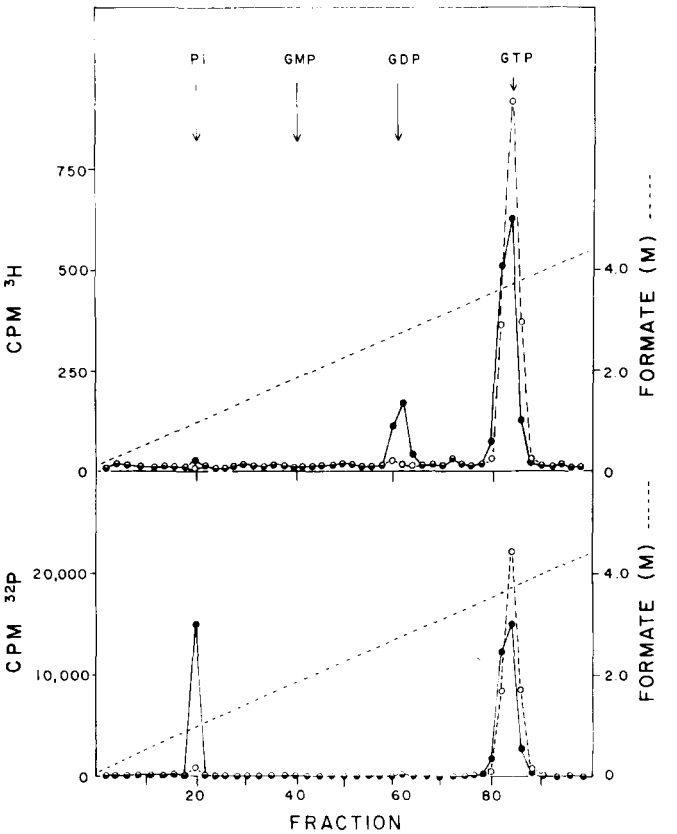

Fig. 2. Dowex-1 formate chromatography of red blood cell membrane GTPase end products after incubation with [^{3}H]GTP (γ-^{32}P). The reaction system contained 0.1 mM GTP, 0.1 M Tris, pH 8.0, 0.5 mM EDTA, 0.01 M $MgCl_2$, and either 0.3—0.35 mg stromal protein (●———●) or a blank without enzyme for control (○———○). The reaction was stopped with 5% trichloroacetic acid, extracted with diethyl ether, and chromatographed on a 0.9 X 32 cm column with a 1 l gradient of 0—5 M ammonium formate/formic acid (4 : 1), pH 3. Fractions were counted for ^{3}H (top) and for ^{32}P (bottom) as described in the text.

M formate. ^{3}H activity was detected in the GTP peak eluting at 3.7 M formate, and in the GDP peak eluting at 2.85 M formate. No other peak of ^{32}P or ^{3}H activity was found, indicating that the phosphate which had been removed from GTP was in the form of inorganic phosphate and the GTP was converted to GDP in the process. Significantly, no peak of radioactivity was found in the GMP region. The recovery of ^{32}P in the inorganic phosphate fraction was incomplete when compared to the amount of GDP recovered from the column, ranging from 49 to 87% of the expected ^{32}P in four experiments. Only a small amount of the ^{32}P could be recovered in the washed trichloroacetic acid-precipitated material, indicating that very little of the phosphate had been transferred to a protein in the protein kinase reaction. When membranes were incubated with GDP at a concentration of 1 mM for 15 and 30 min, no loss of GDP was observed and no GMP was formed, indicating that the system did not contain non-specific phosphatases which could hydrolyze GDP to GMP and that no guanylate kinase activity was present.

TABLE II

EFFECT OF ATP, ADP, CTP AND UTP ON GTPase ACTIVITY OF RED CELL STROMA

Enzyme activity was measured as described in Table I. Inhibition is expressed as % of activity of the control containing no additive. n.t., not tested.

Additive (mM)	Substrate concentration	
	0.2 mM GTP	2.0 mM GTP
None	100	100
ATP 0.2	143	n.t.
0.5	105	n.t.
2	23	64
5	n.t.	32
10	7	n.t.
ADP 0.2	154	n.t.
0.5	181	n.t.
1.0	227	n.t.
CTP 0.2	108	n.t.
0.5	92	n.t.
2	n.t.	90
5	n.t.	69
UTP 0.2	102	n.t.
0.5	88	n.t.
2	n.t.	86
5	n.t.	70

The effect of other nucleotides on GTPase activity

The effect of other nucleoside triphosphates on the cleavage of radioactive GTP by stromal preparations was measured both at a 2 mM and at a 0.2 mM concentration of GTP (Table II). CTP and UTP had very little effect. ATP inhibited the GTPase activity at a high (2 mM) GTP concentration. However, at GTP concentrations of 0.1 mM (data not shown) and 0.2 mM (Table II), the addition of 0.2 mM ATP not only failed to inhibit GTPase activity, but actually appeared to produce stimulation of enzyme activity. ADP exerted even greater stimulatory effect than ATP. It seemed possible that the apparent stimulation of GTPase activity was due to cyclic participation of ATP in the following series of reactions:

$$ATP \xrightarrow{ATPase} ADP + P_i \quad (1)$$

$$GT^{32}P + ADP \xrightarrow{NDPK} GDP + AT^{32}P \quad (2)$$

$$AT^{32}P \xrightarrow{ATPase} ADP + {}^{32}P_i \quad (3)$$

When [γ-^{32}P]GTP was incubated with red cell stroma and ADP, the reaction terminated with trichloroacetic acid and the reaction products chromatographed, radioactive ATP could be recovered. In an incubation system containing 100 μM each of [γ-^{32}P]GTP ($384 \cdot 10^3$ cpm) and 100 μM of unlabelled ADP, the distribution of ^{32}P recovered from the column was as follows: $182 \cdot 10^3$ cpm as $^{32}P_i$; $60 \cdot 10^3$ cpm as [^{32}P]ADP; $48 \cdot 10^3$ cpm as [^{32}P]ATP; and

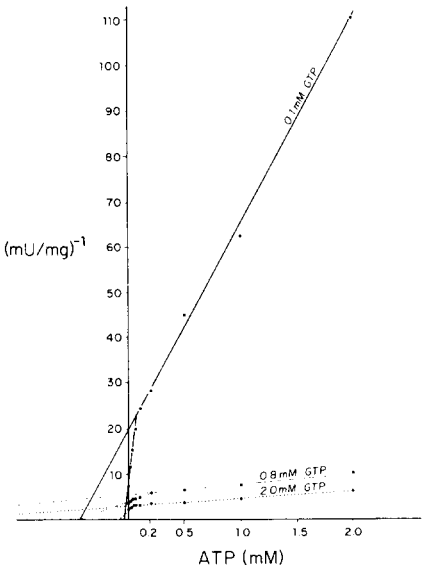

Fig. 3. Dixon plot of inhibition of red blood cell membrane GTPase by ATP. Assay conditions are described in the text. Stroma was washed with 0.5 M urea prior to assay. GTP concentrations were as shown in the figure with ATP ranging from 0 to 2.0 mM.

$94 \cdot 10^3$ cpm as [^{32}P]GTP. The presence of adenylate kinase in the stromal preparation, leads to rapid equilibration of ^{32}P between ADP and ATP. In incubations carried out in the absence of unlabelled ADP, radioactivity is found only in P_i and GTP (see Fig. 2). Accordingly, we concluded that the red cell membranes must contain residual nucleoside diphosphokinase activity, ordinarily considered to be a soluble red cell enzyme [12]. This residual activity could be removed from membranes by washing with 0.5 M urea in 0.01 M Tris, pH 7.4, at 4°C. Using such urea-washed membranes, ATP proved to be a potent competitive inhibitor of GTPase at low concentrations of GTP as well as at high concentrations (Fig. 3). The data did not follow classical kinetics, possibly because a mixture of two enzymes was being studied simultaneously. The data suggest that both types of GTPase have a similar affinity for ATP and GTP, i.e., the addition of non-labeled ATP at any concentration results in a decrease of ^{32}P formed roughly in proportion to the dilution of the labeled GTP with ATP. However, when [γ-^{32}P]ATP was substituted for [γ-^{32}P]GTP in the assay procedure, only a single-component Lineweaver-Burk plot with a K_m value of approx. 0.23 mM was obtained.

Other properties of red cell membrane GTPase

GTPase activity was stimulated 20—50% by 0.5—5 mM GSSG. To determine whether GTPase played a role in outward GSSG transport by red cell membranes [13], 2 mM GSSG was sealed into red cell membranes along with either 2 mM GTP or 2 mM ATP. The membranes containing ATP transported GSSG at a rate of 0.85 nmol/ml membranes per min, compared with a rate of 0.46 nmol/ml membranes per min when GTP had been sealed into the red cells, indicating that GTP was not a preferred energy source for transport of GSSG.

Attempts to solubilize GTPase activity from the membranes were unsuccessful. Extracting the extrinsic proteins from membranes by treating with 0.8 mM EDTA, pH 7.0, overnight at 4°C, failed to remove the GTPase activity from the stroma. Neither did treatment with antispectrin antiserum [14] affect the GTPase activity of red cell membranes. When red cell membranes were treated with 0.5% Triton X-100 in 56 mM sodium borate or 50 mM Tris, pH 8, about 60% of GTPase activity was lost, and only about 1/3 to 1/2 of the remaining activity appeared in the 100 000 × *g* supernatant.

All GTPase activity was destroyed by boiling. The activity had a broad flat pH optimum in the range of 5.5 to 9.0. Both the low and high K_m activities were increased in membranes from a patient with hereditary spherocytosis, presumably because of the young mean red cell age. The GTPase activity at low (0.1 mM) and high (2.0 mM) substrate concentrations, respectively, was 0.51 and 2.8 munits/mg in hereditary spherocytosis red cell membranes while the normal control was 0.38 and 1.3 munits/mg.

Discussion

Membrane-bound red cell GTPase activity is clearly not a non-specific neutral phosphatase, since it requires magnesium and since degradation of GTP only proceeds to the formation of GDP; when GDP was added to membrane fractions containing GTPase activity, no GMP was formed nor was there competitive inhibition of GTPase by GDP.

The reaction appears to be the simple cleavage of the γ-phosphate from GTP:

$$GTP \rightarrow GDP + P_i$$

The amount of phosphate bound to stromal protein for the mediation of protein kinase seems to be negligible in relation to the amount of inorganic phosphorus cleaved from GTP.

Cleavage of high concentrations of GTP by the red cell membrane seems to be catalyzed by a different enzymatic activity than cleavage of low concentrations of GTP. Both the 'high K_m' GTPase and 'low K_m' GTPase of red cell membranes were competitively inhibited by ATP. The data imply that the active sites of these enzymes are relatively non-discriminating with respect to GTP and ATP. We were unable to separate the two enzyme activities from each other, since our attempts to solubilize the enzyme in good yield failed. Spectrin, the myosin-like protein of red cell membranes, apparently was not responsible for an appreciable portion of the GTPase activity. The activity could not be extracted with low concentrations of EDTA and it was not affected by antispectrin antibody. It may be that the low K_m GTPase is the same activity which has sometimes been characterized as the sodium- and potassium-independent ATPase activity of red cell membranes. The identity of the membrane-bound high K_m GTPase activity and its function is not clear.

Acknowledgement

This work was supported, in part, by Grant No. HL 07449 from the National Institutes of Health.

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