ANTISICKLING ACTIVITY OF PHENYLALANINE: EFFECT ON ERYTHROCYTE MEMBRANE ATPASES

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ABSTRACT

The sickling of homozygous sickle cells upon deoxygenation with 2% sodium metabisulphite is inhibited in the presence of phenylalanine (Phe). In the presence of 200 μM Phe there was a rapid reversion of already sickled cells. More than forty percent (46.4 ± 3.2, range 42.4 – 56.2%) sickling reversion could be achieved within 30 min after the introduction of Phe. ATPase activities were studied in erythrocyte membranes prepared from blood of normal, sickle and heterozygous individuals. Phe stimulated Na⁺, K⁺-ATPase activity in both normal and sickle cell ghosts in a concentration dependent manner. Membrane bound Ca²⁺-ATPase activity in normal and sickle cell ghosts was also stimulated by Phe. Phe inhibited Mg²⁺-ATPase activity. Inhibition was found to be non-competitive with a Ki of 1.9 ± 0.08 mM. The antisickling effect of Phe may be related to its effect on the erythrocyte membrane.

KEY WORDS: Phenylalanine; sickled cells, erythrocyte membrane, ATPases.

INTRODUCTION

Increasing interest in the management of sickle cell disease has led to the discovery of many antisickling agents. Certain amino acids (Noguchi and Schechter, 1977) and small peptides (Gorecki et al, 1980) have been shown to produce varying degrees of in vitro inhibition of deoxy-HBs gelation. Effective compounds have been shown to have aromatic acids chains which have a low solubility in aqueous medium. This activity is directly related to the hydrophobicity of the molecules (Gorecki, et al, 1980).

Since the mutation of the β-globin chain of HbA causes a change of glycine to valine, the importance of the hydrophobic binding is not unexpected. Non-polar substances could alter intermolecular interactions involved with a complementary hydrophobic site for β-β valine thereby preventing or delaying gelation of deoxy-HbS (Gorecki et al, 1980).

Ekeke and Shade (1990) reported that phenylalanine is the predominant antisickling agent in Ca janus cajan extract. Phenylalanine and cajanjan cajan extract may be similar in their actions although the extract contains some other amino acids other than phenylalanine.

Although various antisickling agents have been proposed for the management of sickle cell disease, the mechanism of their antisickling effect is not yet clear. This work tends to investigate the effect of phenylalanine on sickling and its possible mechanism of action. The stability of the erythrocyte membrane is achieved by the unidirectional activated transport of ions across the membrane (Kyte, 1971). These vectorial transports are accomplished by the activities of the erythrocyte membrane ATPases.

MATERIALS AND METHODS

Blood Collection

Venous blood samples (5-10ml) were collected into heparinized tubes from both normal and sickle cell anemia individuals. The samples were then separated into 36 AA, 32 AS and 24 SS using cellulose acetate paper electrophoresis.

Ghost Preparation

The erythrocyte membrane ghosts were prepared based on the method of Hamlyn and Duffy (1978). Red cells from fresh human heparinized venous blood were washed three times with 0.5M NaCl (pH 7.4). Membrane was prepared by hypotonic hemolysis in swirling ice-cold 5mM NaH₂PO₄, pH 7.7. The mixture was centrifuged at 5,000g for 1h. The supernatant was removed and the ghost washed once in 10mM Tris-HCl pH 7.7.

The ghosts were later suspended in 3.0ml distilled water. The membranes were stored at 4°C for not more than 12h before being assayed for enzyme activity.
Assay of ATPase Activity

Membrane ATPase activities were estimated by the procedure described by Heesketh et al (1978). ATPase activity was assayed by measuring the inorganic phosphate released from ATP hydrolysis. Inorganic phosphate released in the ATPase assay was determined by the method of Fiske and Subbarow (1925). The activity of the enzyme was expressed as μmole Pi/mg protein/h.

Na⁺,K⁺-ATPase Activity

The enzyme-catalysed reaction was followed spectrophotometrically in a mixture containing 0.5ml of 350mM NaCl, 0.5ml of 17.5mM KCl, 10mM Tris-HCl buffer pH 7.4 and 0.5ml of 2mM ATP-Na₂. Reaction was started by adding 0.2ml ghost cells. The mixture was incubated for 1h at 37°C. Reaction was terminated by adding 0.8ml ice-cold 10% TCA. Tubes were then allowed to stand at 4°C for 5 min, after which they were centrifuged at 4,000g for 5min. To the supernatant was added 1ml of 2.5% ammonium molybdate and after 10min. 1ml of 2.0% ascorbic acid. The tubes were then allowed to stand for 20min. for colour to develop. Absorbance was read at 725 nm using spectronic 21 spectrophotometer.

Ca²⁺-ATPase

The assay medium contained 0.5ml of 21mM MgCl₂, 0.5 ml of 17.5mM CaCl₂, 0.6ml of 10mM Tris-HCl buffer pH 7.4 and 0.5ml of 20mM ATP-Na₂. The reaction was started and terminated as already described for Na⁺, K⁺-ATPase. All assays were run in duplicate.

Mg²⁺-ATPase

Mg²⁺-ATPase activity was assayed spectrophotometrically in a medium containing 0.5ml of 21mM MgCl₂, 0.5ml of 2mM ATP-Na₂, 0.5ml of 10mM Tris-HCl buffer pH 7.4 and 1.0ml distilled water and usually 0.2ml ghost cells. The inorganic phosphate liberated was measured by the method of Fiske and Subbarow (1925).

Determination of Protein

The protein contents of erythrocyte membrane preparation were determined by the method of Lowry et al (1951) with bovine serum albumin (BSA) as standard.

Table 1: Activities of the Membrane-bound Normal and Sickle Cell ATPases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AA (n = 36)</th>
<th>AS (n = 32)</th>
<th>SS (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPases (μmole Pi/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein/h x 10⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>284.7± 20.2</td>
<td>272.4±16.4</td>
<td>118.0±2.8</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>304.2± 4.8</td>
<td>302.6± 4.8</td>
<td>218.4±8.2</td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>144.8± 1.3</td>
<td>108.2± 2.8</td>
<td>234.4±2.0</td>
</tr>
</tbody>
</table>

Values are means ± Standard deviations. The number of samples analysed (n) is given in parentheses.
RESULTS

ATPase activities of sickle and normal RBC membranes are shown in Table 1. Ghosts from AA cells exhibited a higher Na\(^+\),K\(^+\)-ATPase (284.7 ± 20.2 x 10\(^3\) μmole pi/Mg protein/h). The Na\(^+\),K\(^+\)-ATPase activities of AA and AS (272.4 ± 16.4 μmole pi/mg protein/h) cells were significantly different from that of SS (118.0 ± 2.3 μmole pi/mg protein/h).

The Mg\(^{2+}\) - ATPase activity of sickle red blood cell membranes was found to be higher than normal while the Ca\(^{2+}\) - ATPase activity of sickle cell membranes was however, lower than normal. The activity of the Ca\(^{2+}\)-ATPase in sickle erythrocytes was about 71.0% of the normal. There was no significant difference between Ca\(^{2+}\) - ATPase and Mg\(^{2+}\) - ATPase activities in AA and AS cells.

In Vitro Sickness Experiments

2% sodium metabisulphite was able to induce sickness. The mean values of relative percentage sickness with time (min) were plotted (figure 1). In the presence of 2% sodium metabisulphite, there was an initially slow increase in sickness, then followed by a rapid increase. In 30 min, over 30.5 ± 0.4% increase above the intial level of sickled cells was observed.

![Image](https://via.placeholder.com/570)

**Fig. 1: Sickness in the Presence of 30% Sodium Metabisulphite**

**Fig. 2: Sickness inhibition of Phenylalanine of 200 μmole Concentration**

**Effect of Phc on Human Erythrocyte Membrane ATPase Na\(^+\),K\(^+\)-ATPase**

Figure 3 shows the effect of Phe on the activities of the membrane-bound Na\(^+\),K\(^+\)-ATPase in normal human red cells and sickle cell ghosts. The activity of the enzyme was stimulated by Phe in both normal (AA and AS) and SS ghosts and stimulation of the enzyme by Phe was concentration-dependent. The stimulation was observed at concentrations between 0-800 μmole. A maximal stimulation (57.2%) was obtained with AA human erythrocyte Na\(^+\),K\(^+\)-ATPase at 400 μmole Phe compared with a maximal stimulation of 65.7% obtained with the sickle cell membrane ATPase at the same Phe concentration. At 400 μmole Phe, the maximal stimulation obtained with AS ghost Na\(^+\),K\(^+\)-ATPase was 22.6%.

**Ca\(^{2+}\)-ATPase:**

The effect of Phe on the activity of normal and sickle ghosts Ca\(^{2+}\)-ATPase activity is shown in figure 4. Phe stimulated membrane-bound Ca\(^{2+}\)-ATPase activity in normal and sickle cell ghosts. The effect of Phe on Ca\(^{2+}\)-ATPase activity depended on its concentration. Maximum stimulations of 20.0%, 19.3% and 37.2% were obtained with AA, AS and SS human erythrocyte Ca\(^{2+}\)-ATPase respectively at 400 μmole. At concentrations above 400μmole, activity greatly decreased.

**Mg\(^{2+}\)-ATPase**

The effect of different concentrations of Phe on erythrocyte membrane-bound Mg\(^{2+}\)-ATPase is
summarized in figure 5. In the presence of Phe, Mg$^{2+}$-ATPase activity markedly decreased. Inhibition of Mg$^{2+}$-ATPase by Phe was observed in AA, AS and SS ghost at all the concentrations studied. The inhibition by Phe was found to be non-competitive (figure 6). The inhibitor constant $K_i$ was calculated to be $1.9 \pm 0.0$ mM using Dixon's plot (figure 7).

**DISCUSSION**

Phenylalanine is an essential aromatic amino acid. It has significant antisickling activity as demonstrated by visual assays. The reasons why it has this activity are not fully understood. Its amphipathic nature together with its molecular weight are probably responsible for its easy passage through the mosaic lipid protein bilayer of the erythrocyte membrane (Naccache and Shalafi, 1973). However, permeability by itself does not appear to be the only factor in the antisickling activity of phenylalanine. The antisickling effect of phenylalanine suggests that this amino acid might be penetrating the erythrocyte cell membrane. This might explain its effect on the activities of the erythrocyte membrane ATPases. Phenylalanine stimulated Na$^+$.K$^+$.ATPase and Ca$^{2+}$.ATPase and inhibited Mg$^{2+}$.ATPase. This suggests an additional role outside that of the antigelling activity of phenylalanine at low concentrations. Gorecki et al (1980) reported that many amphipathic molecules bind in a biphasic manner to intact erythrocytes. At low concentrations they give linear binding and at higher concentrations they induce sphericity followed by haemolysis. This morphological feature may suggest an interaction between the molecule and
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Fig. 5: Inhibition of Sickle and Normal Ghost Mg²⁺ ATPase Activity by Phenylalanine. ATPase activity was determined as described under "Materials and Method." Except that different concentrations of Phenylalanine were used.

Fig. 6: Lineweaver-Burk plot (1/V vs 1/[S]) at different inhibitor concentrations.

The synergistic effect of Phenylalanine may have led to an increase or decrease in the activities of the erythrocyte membrane ATPases.

REFERENCES


