INHIBITION OF SICKLING BY METHYL ACETIMIDATE

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1. Introduction

A computer generated model recently proposed for the alignment of hemoglobin S molecules in the microtubules which form within deoxygenated sickled cells, has implicated several Lys side chains in the intermolecular contact regions [1]. If this aspect of the model is correct, chemical modification of these amino groups would be expected to inhibit sickling. Imidoesters would appear to be appropriate reagents for this purpose since they have been shown to react selectively with protein amino groups [2,3]. MAI* was employed for the initial in vitro studies because: (1) modification of protein amino groups reportedly does not alter the charge of the protein at physiological pH [4]; (2) reaction of hemoglobin A with MAI does not appreciably affect co-operativity or the Bohr effect [5]; (3) DMA, a bifunctional imidoester, has been shown to inhibit sickling [6]; (4) MAI is the smallest imidoester and therefore should introduce less steric obstruction than any other imidoester.

2. Materials and methods

Fresh heparinized blood was obtained from sickle cell anemia patients. The amount of hemoglobin F, determined by the alkali denaturation method [7], was less than 4%. Erythrocytes were washed with Krebs-Henseleit buffer, pH 7.4, containing 200 mg/100 ml glucose, and were resuspended to a final hematocrit of 20%. One vol of the erythrocyte suspension was diluted with four vols of 0.14 M Tris–HCl buffer, pH 8.8. Equal vols of this suspension were added to flasks containing appropriate amounts of DMA or MAI (Pierce Chemical Co.). Control samples were prepared and treated in an identical manner, omitting the imidoesters. The solutions were incubated for 20 min at room temperature and then washed with Krebs–Henseleit buffer to lower the pH. A small portion of the washed cells was resuspended to a hematocrit of 40%, divided into aliquots and equilibrated with either water-saturated 5% CO_2–95% N_2 or 5% CO_2–95% air gas mixtures for 1.5 h. The gas equilibrated solutions were transferred anaerobically into 10% (v/v) HCHO in 0.9% saline for fixation. The percentage of sickled cells in a total of 500 cells was determined. The main portion of the incubated cell suspension was lysed according to the method of Drabkin [8], and dialyzed overnight at 4°C against 0.15 M potassium phosphate buffer, pH 7.35. Lysate obtained from treated erythrocytes was subjected to starch gel electrophoresis [9], gel filtration on Ultrogel AcA 44 (LKB), and the MGC of each treated sample was determined in triplicate and expressed as the range in values, according to the method of Bookchin et al. [10]. The dialyzed samples were concentrated by centrifugation through Centriflo membrane cones (Amicon). The percentage of methemoglobin was determined spectrophotometrically [11].

* *Abbreviations:* MAI, methyl acetimidate; DMA, dimethyl adipimidate; MGC, minimum deoxygenated hemoglobin concentration required for gelation.

3. Results

Incubation of sickle cell anemia erythrocytes with increasing concentrations of MAI resulted in a progressive decrease in the number of sickled cells both after complete deoxygenation (table 1) and in the presence of air (table 2).

An increase in MGC was observed in lysate obtained from the MAI treated erythrocytes. The MGC of dialyzed lysate obtained from untreated erythrocytes was 26.1 ± 0.4 gm%, and increased to 28.6 ± 0.4 gm% and 34.5 ± 0.2 gm% after the cells were incubated with 5 and 10 mM MAI, respectively. Since the methemoglobin level in these cells was below 1%, there is no question that the antisickling effect of MAI was due to chemical modification of the hemoglobin. The MGC value of erythrocytes treated with 5 mM DMA could not be determined because these cells could not be lysed even when four volumes of water and one volume of toluene per volume of packed cells was used. The small amount of hemoglobin that was obtained formed a gel upon standing in the presence of oxygen. Similar results have been reported for DMA treated normal human erythrocytes [12]

Gel filtration of lysate obtained from MAI incubated erythrocytes (sickle as well as normal) on Ultrogel AcA 44 (1.2 X 86 cm) in 0.15 M potassium phosphate at pH 7.35, flow rate 0.6 ml/min, revealed the presence of hemoglobin components eluting ahead of normal hemoglobin in the void volume of the column. The amount of high mol. wt. material varied as a function of the MAI concentration used in the incubation (table 3).

Table 1
Effect of imidoesters on erythrocyte sickling upon complete deoxygenation

Imidoester	% Sickled cells
None	91.0
5 mM DMA	5.3
5 mM MAI	5.0
10 mM MAI	3.0

For reaction conditions see Materials and methods.

Table 2
Effect of imidoesters on irreversibly sickled cells

Imidoester	% Sickled cells
None	5.8
5 mM DMA	4.2
5 mM MAI	2.7
10 mM MAI	1.5

Table 3
Proportion of high mol. wt. hemoglobin S components as a function of MAI concentration[a]

MAI (mM)	% High mol. wt. HbS
1.25	14.6
2.5	18.4
5.0	22.6
10.0	26.9

[a] Dialyzed lysate obtained from MAI incubated sickle cell anemia erythrocytes was gel filtered on an Ultrogel AcA 44 column (1.2 X 86 cm) equilibrated with 0.15 M potassium phosphate, pH 7.35.

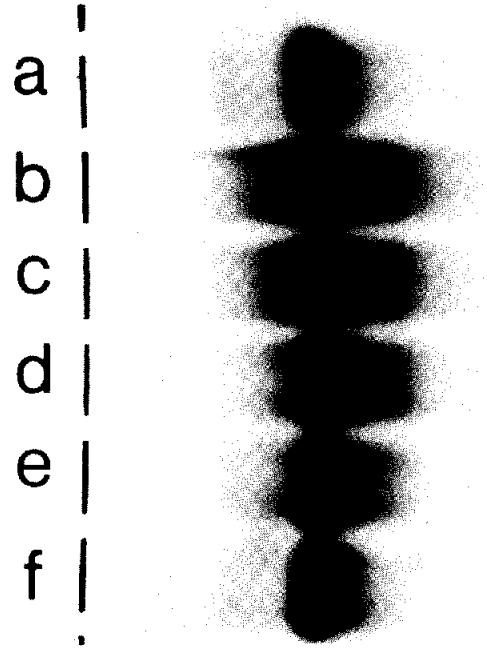

Fig.1. Starch gel electrophoresis in Tris-borate-EDTA buffer, pH 8.6, of lysate obtained from erythrocytes treated with MAI; stained with amido black. Erythrocytes obtained from sickle cell anemia patients were treated with: (a) and (f) 0 (Control), (b) 10 mM, (c) 5.0 mM, (d) 2.5 mM, and (e) 1.25 mM, MAI.

Starch gel electrophoresis with Tris-borate-EDTA buffer, pH 8.6, of lysate obtained from the MAI treated erythrocytes showed components moving both more rapidly and more slowly toward the anode than the principal component (fig.1). The chemical nature of the components with altered charge has not been determined. Starch gel electrophoresis of the high mol. wt. material obtained after gel filtration showed it to be the slow moving components.

4. Discussion

MAI has been shown to inhibit sickling of sickle cell anemia erythrocytes after complete deoxygenation. The number of irreversibly sickled cells was also decreased. Although the molecular basis for this inhibition has not yet been established, reaction of hemoglobin A with MAI at a higher hemoglobin: MAI molar ratio (1 : 10) has been shown [5] to modify 1% of the valyl N-terminal residues and 3% of lysyl residues in the α chain, and 3% of the N-terminal valyl residues and 14% of the lysyl residues in the β chain. The oxygen affinity of the modified hemoglobin A was increased, but a change in oxygen affinity cannot account entirely for the decrease in sickling reported here, since the effect was observed in the absence of oxygen. The inhibition of sickling possibly is due to destabilization of the deoxyhemoglobin S aggregate caused by modification of Lys residues involved in the intermolecular contact regions, and/or to modification of the N-terminal amino groups.

DMA also has been shown to inhibit sickling after complete deoxygenation of treated erythrocytes, and high mol. wt. components were observed as a function of DMA concentration [6]. Contrary to previous results on proteins treated with monofunctional imidoesters at low protein concentrations [2,4,13] high mol. wt. components were also observed in the MAI treated erythrocytes. However, reaction of 4.97 mM hemoglobin S lysate with 9.35 mM MAI after dialysis against 0.14 M Tris–HCl buffer, pH 8.8 at 4°C overnight, gave only 1.2% high mol. wt. components. Apparently, formation of high mol. wt. hemoglobin components is dependent upon particular conditions found in the red cell. The relationship between the high molecular weight components and the antisickling effect of MAI is yet to be determined.

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