REVERSIBLE SOLUBILITY OF DEOXYHEMOGLOBIN S

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SUMMARY

The solubility of deoxyhemoglobin S in 1.96 M phosphate is sensitive to changes in oxygenation and temperature in a manner similar to the widely used in vitro gelation assay. In addition, the pH of the phosphate buffer used in the solubility determination has a profound effect on deoxyhemoglobin S solubility. It is suggested that solubility in 1.96 M phosphate may be a sensitive method of monitoring the aggregation phenomenon of deoxyhemoglobin S.

Itano showed in 1953 that deoxy Hb S formed an amorphous precipitate in 2.24 M phosphate buffer, pH 7.0, while oxy Hb S and oxy- and deoxy Hb A did not (2). In the present study we have attempted to determine whether the solubility of deoxy Hb S responds to oxygen and temperature in a manner consistent with results reported from in vitro gelation studies and sickling studies with erythrocytes containing Hb S (3,4,5). A set of conditions is described for measuring the solubility of deoxy Hb S which is sensitive to the above parameters and suggests that solubility is a useful method for quantitating the aggregation phenomenon of deoxy Hb S.

MATERIALS AND METHODS

Samples of blood from individuals either heterozygous or homozygous for Hb S were obtained from the Hematology Service of the Department of Internal Medicine, The University of Texas Southwestern Medical School. The hemoglobin was isolated from red cells via the procedure of Drabkin (6) and disc gel electrophoresis was carried out on these isolated hemoglobin samples to determine their homogeneity. Samples of electrophoretically homogeneous Hb S were used without further purification. Hb S was isolated from electrophoretically heterogeneous hemoglobin hemolysates by ion exchange chromatography on

carboxymethyl cellulose (Whatman CM 52) which had been equilibrated with 10 mM phosphate buffer, pH 6.0. Elution was carried out by using a gradient of 10 mM phosphate buffer, pH 6.7 to 20 mM phosphate buffer, pH 7.5. Column fractions containing only Hb S, as judged by disc gel electrophoresis, were pooled and concentrated before use. In all cases disc gel electrophoresis was carried out as described by Ornstein and Davis (7,8) using 7.5% cross-linked polyacrylamide gels, Tris-glycine buffer, pH 8.6, and 4 mamps per tube of 4 mm diameter.

The solubility tests in 2.24 M potassium phosphate buffer, pH 7.0, were carried out as described by Itano (2) using a total volume of 1.0 ml which contained 10 mg of $Na_2S_2O_4$ and 2.0 mg of hemoglobin. All reagents were equilibrated at the temperature stated, mixed, and incubated for 15 minutes. The reaction mixture was then filtered using a five micron Millipore filter and the heme concentration in the filtrate was determined by measuring the absorbance at 419 nm, after reduction with $Na_2S_2O_4$ and bubbling with CO (ε = 191 $mM^{-1}cm^{-1}$) (9). The effect of phosphate buffer concentration was determined by using different size aliquots of 2.8 M potassium phosphate buffer, pH 7.0 and enough water to give a final reaction mixture of 1 ml. The effect of temperature on the solubility of deoxy Hb S was examined both by incubating the standard reaction mixture at 4° instead of 37° and by cooling the standard reaction mixture to 4°C after the 15 minute incubation at 37°C. Reoxygenation of insoluble deoxy Hb S was accomplished within 30 minutes at room temperature by stirring while passing a stream of oxygen gas over the top of the solution. The effect of pH on the solubility of deoxy Hb S was determined by using 1.96 M potassium phosphate buffer at different pH values. The solubility of mixtures of hemoglobins S and A was measured in 1.96 M potassium phosphate buffer, pH 7.0, by keeping the Hb S concentration constant at 2 mg/ml and varying the concentration of hemoglobin A from 1 mg/ml to 4 mg/ml.

RESULTS AND DISCUSSION

Table I contains the results obtained when the solubility of Hb S is examined

TABLE I

HEMOGLOBIN SOLUBILITY IN PHOSPHATE BUFFERS*

HEMOGLOBIN SAMPLE	PHOSPHATE (M)	INCUBATION TEMPERATURE		TEMPERATURE REVERSAL	OXYGEN REVERSAL (37°C)
		37°C	4°C		
Deoxy Hb S	2.24	0.13 ± 0.12 (17)	0.61 ± 0.08 (20)	0.74 ± 0.2 (4)	0.86 ± 0.27 (7)
	1.96	0.11 ± 0.12 (27)	1.81 ± 0.18 (10)	1.37 ± 0.23 (6)	1.22 ± 0.26 (9)
Oxy Hb S	2.24	1.68 ± 0.28 (4)	1.75 ± 0.05 (4)	-	-
	1.96	1.64 ± 0.20 (10)	1.51 ± 0.16 (3)	-	-
Deoxy Hb A	2.24	1.83 ± 0.21 (11)	1.97 ± 0.21 (12)	-	-
	1.96	1.79 ± 0.22 (11)	1.96 ± 0.18 (4)	-	-
Oxy Hb A	2.24	1.98 ± 0.05 (5)	1.95 ± 0.06 (7)	-	-
	1.96	1.97 ± 0.10 (6)	1.95 ± 0.13 (6)	-	-

* All values are expressed as mg hemoglobin per ml remaining in the filtrate and are given as the mean value, standard deviation and number of experiments.

Initial reaction mixtures contained 2.0 mg of protein, the noted concentration of potassium phosphate buffer, pH 7.0, and 10 mg of $Na_2S_2O_4$ in a total volume of 1.0 ml.

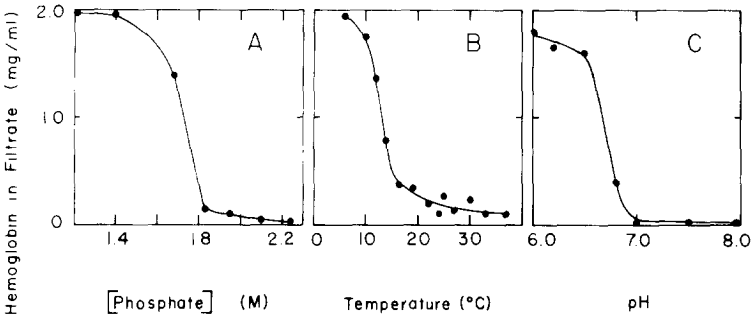

Figure 1: Effect of phosphate buffer concentration (A), incubation temperature (B), and pH (C) on the solubility of deoxyhemoglobin S. The standard reaction mixtures (1.0 ml total volume) contained 2.0 mg of protein, 10 mg of $Na_2S_2O_4$ and 1.96 M phosphate buffer, pH 7.0, and were incubated for 15 minutes at 37° prior to filtration with 5 micron Millipore filters. In (A) the concentration of phosphate buffer was varied, (B) the incubation temperature was varied, and (C) the pH of the phosphate buffer was varied.

in 2.24 M and 1.96 M phosphate buffer. With these two phosphate buffer concentrations no significant decrease in solubility of oxy Hb S, deoxy Hb A or oxy Hb A upon incubation at 37° is seen. In contrast, under these conditions deoxy Hb S is almost completely insoluble. Incubation at 4°C in 2.24 M phosphate buffer increases the amount of deoxy Hb S remaining in solution after filtration as does lowering the temperature to 4° prior to filtration after incubation at 37° for 15 minutes. Oxygenation of the deoxy Hb S reaction mixture after incubation at 37° for 15 minutes also results in an increase in hemoglobin remaining in the 2.24 M phosphate buffer filtrate.

The increase in the solubility of deoxy Hb S upon oxygenation or upon decreasing the temperature suggests that the insolubility is reversible and therefore could be reflecting the same phenomenon as the gelation assay. For this reason, the conditions of the solubility measurement were altered to obtain the maximal increase in solubility of deoxy Hb S upon oxygenation or upon decreasing the temperature.

When the effect of phosphate buffer concentration on the solubility of deoxy Hb S was examined, the results in Fig. 1A were obtained. As the concentration of phosphate buffer, pH 7.0, was decreased from 2.24 M to 1.96 M the solubility of deoxy Hb S was constant. Decreasing the phosphate concentration below 1.96 M

dramatically increased the solubility of deoxy Hb S until at a concentration of 1.4 M phosphate buffer deoxy Hb S was completely soluble. The studies in 2.24 M phosphate were therefore repeated using 1.96 M phosphate buffer, pH 7.0 with the results shown in Table I. Incubation at 4°C gives a three fold increase in the amount of deoxy Hb S remaining in solution after filtration as compared to the data in 2.24 M phosphate buffer. Likewise, in 1.96 M phosphate buffer, a two fold increase in the solubility of deoxy Hb S is obtained by lowering the temperature to 4° prior to filtration after incubation at 37°C for 15 minutes. Oxygenation of the deoxy Hb S reaction mixture in 1.96 M phosphate buffer after incubation at 37° for 15 minutes, also results in an increase in hemoglobin remaining in the filtrate. The reason that deoxy Hb S is not more soluble upon oxygenation may be due to difficulty in completely oxygenating hemoglobin in the insoluble form. The data in Table I shows that while 1.96 M phosphate is just as effective as 2.24 M phosphate in discriminating between deoxy Hb S and oxy Hb S, it is much more effective in demonstrating the reversibility of deoxy Hb S solubility as a function of temperature or oxygenation.

The effect of temperature on the solubility of deoxy Hb S in 1.96 M phosphate buffer, pH 7.0, is shown in Fig. 1B. These results indicate that deoxy Hb S remains soluble at incubation temperatures lower than 6°C. As the incubation temperature is increased, a very sharp decrease in the solubility of deoxy Hb S occurs between 10° and 16°C. At incubation temperatures above 16°C the maximum insolubility of deoxy Hb S is obtained. A similar sharp change between 10°-16°C was observed by Allison when measuring the specific viscosity of deoxy Hb S solutions at concentrations of 146 mg/ml and higher (5).

The effect of pH on the solubility of deoxy Hb S in 1.96 M phosphate buffer is seen in Fig. 1C. It can be seen that above pH 7.0 the deoxy Hb S is completely out of solution. As the pH is lowered to values below 7.0 the solubility of deoxy Hb S increases dramatically and at pH 6.4, 80% of the deoxy Hb S is soluble under these assay conditions. These results show that the solubility of deoxy Hb S in 1.96 M phosphate is responsive to changes in the physical properties of the protein.

This increase in solubility with decrease in pH could be due to changes in the number of ionizable groups on the protein or to a conformational change in the protein which alters the groups exposed to the solvent environment.

The solubility of Hb S in 1.96 M phosphate buffer is quantitative enough to allow an estimation of the amount of Hb S in mixtures containing both Hb S and Hb A. Addition of up to 4.0 mg of Hb A to the reaction mixture does not change the amount of Hb S found in the filtrate. The hemoglobin in the filtrate is more than 95% Hb A as determined from disc gel electrophoresis. This experiment shows that solubility in 1.96 M phosphate easily separates mixtures of hemoglobins S and A and therefore suggests that large scale separation of the two hemoglobins is possible by this method. In addition, solubility in 1.96 M phosphate could be used to determine whether an individual is homozygous or heterozygous for Hb S.

The importance of the reported solubility assay as compared to the standard Itano test is that it permits the use of small amounts of hemoglobin (2 mg total protein), is sensitive to changes in temperature and oxygenation, and is responsive to changes in the physical properties of Hb S. Whether solubility measurements reflect the same properties of deoxy Hb S as the *in vitro* gelation assay or the sickling of erythrocytes remains an open question and is currently under investigation. However, the fact that the temperature dependence of solubility and viscosity of deoxy Hb S are similar suggests that this may be the case and this coupled with the oxygenation and pH studies indicates that solubility may be a good method of monitoring deoxy Hb S aggregation.

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