Optimization of Reaction Conditions for the Preparation of Subunits from Variant Hemoglobins

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Introduction

Several reports exist in the literature about the effectiveness of sodium *para*-chloromercuribenzoate and other organomercurials as inducers of dissociation of normal human adult hemoglobin (2,6,8). These organomercurials have been shown to be selective in reacting with protein thiol groups. Once an organomercurial reagent is covalently linked to the reactive thiols in the beta subunits (cysteinyl residue 3), symmetrical dissociation to dimers occurs. Additional reagent is linked to cysteinyl residue 112 in the beta subunits and to cysteinyl residue 104 in the alpha subunits to complete dissociation to isolated hemoglobin subunits (6,7).

The organomercurials can be removed by a variety of mild conditions to yield subunits whose physical and chemical properties have been characterized (3,1). These subunits can recombine to form stable tetramers with biological and physicochemical properties that are identical to normal adult hemoglobin (1).

Unfortunately, no mention is made of yield of isolated subunits in the above references. This question is of importance when attempting to isolate subunits from variant hemoglobins. (Variant hemoglobin subunits can be used in physicochemical studies assessing influence of primary structure on quarternary structure and in the preparation of purified antisera with diagnostic potential.) Thus, the effects of incubation temperature, time of incubation, organomercurial structure, and incubation pH on overall yield of soluble hemoproteins remaining in a reaction mixture have been investigated with hemoglobin A, and information obtained has been applied to the preparation of isolated subunits from Hemoglobin S.

Materials and Methods

Hemoglobin was isolated from freshly drawn saline washed erythrocytes by the water-toluene lysis method (5) and was stored at 5° C in the CO saturated form.

One gram of hemoglobin (determined by absorbance of the CO derivative at 540 mm) was diluted to a final volume of 10 ml with a final buffer concentration of 0.01M sodium phosphate, 0.1M sodium chloride. Temperature was regulated during the experiments with a Temp-Stir temperature regulator (Precision Scientific Co.). Organomercurials (purchased from Sigma Chemical Co.) were dissolved in the minimum volume of 1M sodium hydroxide and adjusted to pH6.0 with 1M hydrochloric acid; final

amount of organomercurial added was 8:1 molar excess compared to hemoglobin. These compounds were rapidly mixed with a hemoglobin solution and timing of incubation was initiated.

At four hour intervals, a representative aliquot was taken from the reaction mixture and centrifuged at 2000 RPM in a Sorvall GLC-1 centrifuge (Sorvall 513/539 rotor) for ten minutes. An appropriately diluted sample of the supernatant was bubbled with CO and absorbance was measured at 540nm with a Beckman Acta C—III spectrophotometer. The percentage of total hemoproteins remaining in this supernatant fluid was finally calculated.

Dissociation into subunits was monitored by cellulose acetate electrophoresis of a 5 microliter sample taken from each supernatant fluid. A Buchler Instrument Co. 3-1014A power supply and a Gelman electrophoresis chamber were used under the following conditions:

Tris-glycine buffer, pH9.3; 5° C; 300 VDC-constant voltage mode; 30 minutes development time; staining in 0.2% Ponceau S (Sigma Chemical Co.) dissolved in 4% trichloroacetic acid; destaining in 5% acetic acid washes (8).

TABLE 1. *Effect of Reaction Temperature on Hemoglobin A Denaturation (PCMB, pH6.0, 0.1M NaCl)*

Reaction Time (In Hours)	Percent Soluble Hemoprotein Remaining	
	at 23° C	at 37° C
4	83	56
8	75	43
12	66	33
16	63	24
20	61	16
24	60	9

Results

The effects of incubation temperature on hemoglobin A denaturation using sodium *para* chloromercuribenzoate (PCMB) as a typical organomercurial is presented in Table 1. It can be seen that there is a profound loss of soluble hemoproteins from the reaction mixtures at both 23° C and 37° C and that this loss increases with incubation time, especially at 37° C. Cellulose acetate electropherograms also reveal that there is no improvement in efficiency of dissociation into subunits at the elevated temperatures. From the viewpoints of both dissociation efficiency and maintenance of subunit yield, 5° C remains the preferred incubation temperature.

Table 2 shows the effect of organomercurial structure on hemoglobin A denaturation. It is seen that the organomercurials used in the present case are not markedly different in magnitude as perturbants of protein conformation. Cellulose acetate electrophoresis also reveals little difference among the compounds in dissociation efficiency. This is not surprising since the principal difference in structure is the replacement of a *para* carboxyl group (in PCMB or PHMB) with a *para* sulfonic acid moiety (in PCMPS or PHMPS). This is in agreement with the findings of Stefanini *et. al.* for dissociation efficiency with

TABLE 2. *Effect of the Organomercurial Structure on Hemoglobin A Denaturation (5° C, 0.1 M NaCl, 0.01 M sodium phosphate, pH6.0)*

Reaction Time (In Hours)	Percent Soluble Hemoprotein Remaining			
	PCMB	PHMB	PCMPS	PHMPS
4	92	94	99	—
8	95	—	100	100
16	97	—	—	97
20	86	93	99	97

other organomercurials. They also concluded that presence of a *para* carboxyl substituent was not essential (8). (These abbreviations refer to the sodium salts of the following chemical compounds: PCMB, *para* chloromercuribenzoate; PHMB, *para* hydroxymercuribenzoate; PCMPS, *para* chloromercuriphenyl sulfonate; and PHMPS, *para* hydroxymercuriphenyl sulfonate).

Hemoprotein denaturation is much greater when incubation occurs at pH6.0 than when pH7.0 is selected (Table 3). Unfortunately, cellulose acetate electrophoresis reveals that very little dissociation into subunits occurs at pH7.0. Dissociation efficienty at pH6.0 has been documented elsewhere also (3,6,8).

Under the present experimental conditions, it has also been observed that dissociation is effective at time intervals less than the 16-20 hour interval commonly used and that denaturation loss is also avoided in this way.

TABLE 3. *Effect of pH of the Reaction Mixture on Hemoglobin A Denaturation (PCMB, 5° C, 0.1 M NaCl, 0.01 M sodium phosphate)*

Reaction Time (In Hours)	Percent Soluble Hemoprotein Remaining	
	at pH 6.0	at pH 7.0
4	89	100
8	87	100
12	84	98
16	77	97
20	72	96
24	66	96

Discussion

From the present studies, the following experimental conditions have been selected for the optimum yield of native hemoglobin A subunits: 0.01 M sodium phosphate buffer, pH6.0; PCMB (or PHMB)/hemoglobin, 8:1 molar excess; incubation temperature, 5°C; and incubation time, 8 hours.

Age of the hemoglobin source is also an important consideration from the veiw point of susceptibility to denaturation. For this reason, stored whole blood samples have been judged unsatisfactory for the present purpose.

Using the above reaction conditions, several successful attempts to isolate β^s subunits from hemoglobin S have occurred. Some rapidly eluted

hemoprotein contaminant has been present in small amounts when the carboxymethyl cellulose chromatographic method of Bucci and Fronticelli has been employed (2). At the present stage of the investigation, no additional information has been obtained about efficiency of removal of the organomercurial, but the use of organomercurial dissociation of variant hemoglobins to yield native abnormal subunits for various uses appears promising.

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