AFFINITY CHROMATOGRAPHY : SPECIFIC BINDING OF HEMOGLOBIN ON AGAROSE LINKED HAPTOGLOBIN

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SUMMARY : Human haptoglobin was coupled to agarose and used as affinity adsorbant to bind human hemoglobin. The optimal conditions of hemoglobin binding and dissociation were defined. It was found that the haptoglobin adsorbant removed effectively and specifically free hemoglobin from hemolysed sera.

Current information on molecular interactions between haptoglobin and hemoglobin has been obtained by studying the whole system in solution. It seemed of interest to explore this interaction when one of the proteins was rendered insoluble. Such a novel approach, besides its theoritical implications could offer immediate practical applications, such as the specific removal of free hemoglobin from hemolysed sera. We report here our first results on affinity chromatography using haptoglobin-agarose as affinity adsorbant for hemoglobin.

EXPERIMENTAL

Human haptoglobin of genetic type 1-1 (Hp 1-1) was obtained from unhemolysed plasma of a homozygous donor and was purified according to Waks and Alfsen (1). The preparation was almost pure, when tested by acrylamide gel electrophoresis according to Ornstein and Davis (2,3), and by immunoelectrophoresis using a potent polyvalent rabbit antiserum raised against normal human serum. The protein concentrations were obtained by spectrophotometric measurements, using an absorption coefficient of $E^{1\%\ 1\ cm}_{280\ m\mu} = 12.0$. The molecular weight of haptoglobin was assumed to be

85,000 Daltons. Hemoglobin binding capacity of the purified Hp 1-1 was measured by fluorescence quenching titration as described by Chiancone et al. (4), using a F.I.C.A. spectrophotometer, and was found to be 90 % of the theoritical value.

Human hemoglobin A_1 (Hb A_1) was purified from lysates of normal human erythrocytes by C.M-Sephadex 50 (Pharmacia) chromatography and starch gel block electrophoresis at pH 8.6 according to Rosa (5). Hemoglobin A_1 was pure when tested by cellulose acetate zone electrophoresis at 104 mg/ml. Hemoglobin concentrations were determined by the cyanmethemoglobin spectrophotometric technique (6). The molecular weight of human HbA_1 was assumed to be 64,500 Daltons.

I^{125} iodination of HbA_1 was performed by the lactoperoxidase technique as described by Marchalonis (7), using carrier-free sodium iodide. The specific activity of I^{125} HbA_1 was measured in a Nuclear Chicago gamma counter and was found to be 0.5 10^{12} c/min/M.

Preparation of haptoglobin-agarose adsorbant.

The method adopted for the cyanogen bromide activation of agarose was that of Porath (8) and Cuatrecasas (9). Settled agarose (Sepharose 4B, Pharmacia) was activated by cyanogen bromide at pH 11-11.5, until cessation of proton release. Haptoglobin in 0.1 M bicarbonate buffer, pH 8.9, was immediately mixed with the washed activated agarose. The mixture was gently stirred at 4°C overnight. The adsorbant was extensively washed with 0.1 M phosphate buffer, pH 7.4, in 0.15 M NaCl and then equilibrated with 0.01M potassium acetate buffer, pH 5.0, in 0.09 M KCl.

The quantity of haptoglobin linked to agarose was established by Kjeldahl determination of the nitrogen content of aliquots of one ml of sedimented material dried to constant weight.

In preliminary experiments, we compared the binding efficiency of the adsorbant either batchwise or using small columns. We found that the latter technique resulted repeatedly in 30 % higher binding efficiency. Therefore we adopted the batchwise technique throughout the study in the following way : to one ml of settled haptoglobin-agarose adsorbant equilibrated in 0.01 M potassium acetate buffer, pH 5.0, in 0.09 M KCl, aliquots of hemoglobin A_1 were

added and gently mixed at room temperature for 15 min. The mixture was then transferred to a chromatography column (10 cm x 0.6 cm) and unbound Hb was washed out, using the same buffer.

Washed activated agarose was prepared in the same manner, but omitting the addition of Hp 1-1. This material was used as a control for nitrogen determination and for an estimation of the non specific binding of the adsorbant after spontaneous desactivation (9).

RESULTS AND DISCUSSION

1. Conditions of haptoglobin coupling to the agarose matrix

Aliquots of sedimented agarose were activated with increasing quantities of cyanogen bromide (100 mg, 200 mg and 300 mg per ml of sedimented agarose) and reacted at pH 8.9 with an equal volume of Hp 1-1 solution at a concentration of 5 mg/ml. As shown in table I, the quantity of Hp 1-1 fixed to the matrix was on one hand increased with the cyanogen bromide concentration and, on the other hand, was further increased when using a haptoglobin solution at 10 mg/ml. These results are in accordance with the findings of Cuatrecasas (9). A further increase in protein concentration, although not investigated in our study, might result in a higher Hp linkage.

2. Hemoglobin binding capacity of agarose-linked haptoglobin

In order to establish the optimal pH conditions for Hb-Hp interactions, I^{125} HbA_1 was added in a ten fold molar excess over the quantity of Hp 1-1 linked to one ml of conjugated agarose, assuming that the binding capacity of insolubilized Hp 1-1 was fully retained. Hemoglobin binding capacity was measured at 3 different pH (0.1 M acetate buffer, pH 5.2 ; 0.1 M borate buffer, pH 7.4; 0.1 M bicarbonate buffer, pH 8.9). In a typical experiment, it was found that 2.6 nM of HbA_1 were bound at pH 5.2, 1.95 nM at pH 7.4 and 1.65 nM at pH 8.9. These findings are in agreement with other data on Hp-Hb interaction in solution. Because of acid-induced denaturation of hemoglobin and haptoglobin, lower pH were not investigated in this study.

By comparing in repeated experiments the binding capacity of soluble Hp 1-1, as measured by fluorescence quenching, with the maximum binding capacity of ensolubilized haptoglobin, it was found that 40 % of the binding

Haptoglobin concentration \ Quantity of BrCN per ml of sedimented agarose	100 mg	200 mg	300 mg
5 mg/ml	13 nM(*)	15 nM	35 nM
10 mg/ml	-	-	62 nM

Table I.

(*) nM of haptoglobin bound per ml of conjugated agarose when activated with increasing quantities of BrCN. The quantity of bound protein was estimated by the following formula :

$$\text{Number of nM} = 6.25 \times (\text{mg of N per ml of dried conjugated} - \text{mg of N per ml of dried activated agarose}) / 85.10^{-3}$$

capacity of the soluble form was retained. This loss in activity might be due to Hp 1-1 denaturation by high pH during the coupling phase or to the unaccessibility of the binding sites once the molecule is fixed on the matrix.

$I^{125}HbA_1$ - Hp 1-1 soluble complexes were prepared in Hb excess (2 : 1 ratio) and were purified by gel filtration on Sephadex G 200. When aliquots of radioactive Hb-Hp complexes, counting 2,210 c/min, were applied on one ml of Hp-adsorbant, 97 % of the total radioactivity was recovered in the effluent. These data indicate that Hb-Hp complexes present in hemolysed sera will not be removed by this method. This experiment also demonstrates that, once the Hb-Hp complex is formed, the binding energy is so high that the solid state Hp 1-1 could not displace the Hb molecules from the complexes.

3. Specificity of the haptoglobin-agarose adsorbant

When one ml of normal human serum was applied to one ml of the adsorbant, the immunoelectrophoretic pattern of the eluted serum was unchanged, when tested against a polyvalent rabbit antiserum at the same protein concentration as that of the unabsorbed serum (70 mg/ml). Furthermore, the levels of IgG, IgA and IgM as measured by Mancini's technique were not decreased after adsorption. Similarly, serum haptoglobin content, when estimated by immunoelectrophoresis using a goat monospecific antiserum, was roughly unchanged after the adsorption step.

4. Dissociation of hemoglobin from agarose-linked haptoglobin

The optimal conditions for the dissociation of HbA_1 bound to the Hp-agarose bed were studied in order to obtain an effective regeneration of the affinity adsorbant. One ml aliquots of adsorbant loaded with $I^{125}HbA_1$ were percolated with the following solvants : 1 M acetic acid, 0.1 M glycin-HCl buffer, pH 2.4 ; 3 M NaSCHN ; 8 M urea ; 5 M guanidine-HCl and 50 % (v/v) dioxane in water. The best recovery (90 % of the bound $I^{125}HbA_1$) was obtained with 5M guanidine HCl whereas glycine-HCl buffer eluted only 60 % of the bound radioactivity. The other reagents were found to be almost ineffective. The binding capacity of the regenerated adsorbant was tested and found to be only 60 % of the initial value.

In conclusion, the proposed affinity adsorbant represents an effective and specific method for binding free hemoglobin. As hemoglobin interferes with several biochemical and immunological reactions, such as complement fixation tests, this adsorbant is useful for removing it from hemolysed sera. Furthermore, preliminary data from this laboratory indicate that the use of haptoglobin (or inversely hemoglobin) agarose adsorbant offers a promising approach for the study of the hemoglobin-haptoglobin interactions, and for the location of the binding sites on both molecules.

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