PREPARATION AND PROPERTIES OF A HEMOGLOBIN CONTAINING HEME ONLY IN GAMMA SUBUNITS

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SUMMARY

A semihemoglobin containing prosthetic groups only in the γ-subunits (two hemes per tetramer) has been prepared by mixing together apo-α-subunits from hemoglobin A and native, heme-containing γ-subunits from hemoglobin F. The semihemoglobin has optical properties similar to those of hemoglobin F. The oxygen affinity of the semihemoglobin is lower than that of isolated γ-subunits but not as low as that of hemoglobin F, and the Hill coefficient of the semihemoglobin is near one. This semihemoglobin lends further support to the non-equivalence of the subunits in the hemoglobin tetramer.

INTRODUCTION

Modification of the protoheme prosthetic group in one type of hemoglobin subunit can offer the opportunity of independently studying the functional properties of the native protoheme group in the other type of hemoglobin subunit. Studies have recently been carried out on modified hemoglobins which contain prosthetic groups only in the alpha subunits or beta subunits (1,2, 3,4). This type of hemoglobin has been called semihemoglobin and has been described by various symbols. The semihemoglobin offers an opportunity to study the properties of one type of subunit in a tetrameric configuration without interference from the other type of subunit.

We have prepared a semihemoglobin consisting of apo-alpha subunits from hemoglobin A and heme-containing gamma subunits from hemoglobin F and the properties of this molecule are reported herein.

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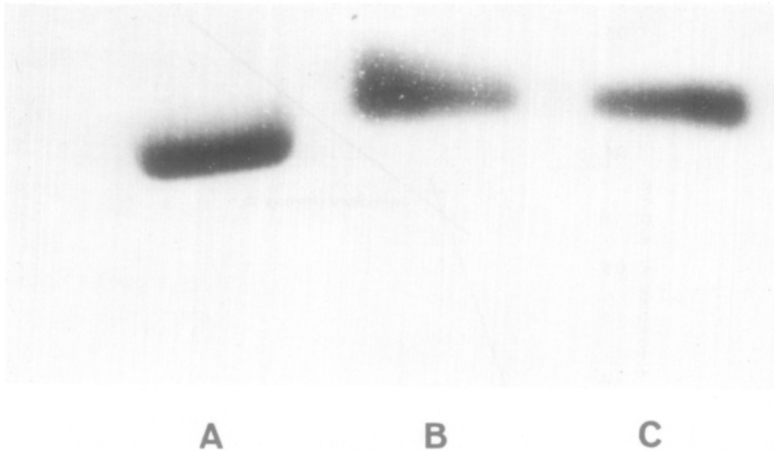

Figure 1: Starch gel electrophoresis pattern of semihemoglobin. (A) isolated γ-subunits, (B) $\alpha_2\gamma_2$ semihemoglobin, (C) native hemoglobin F. The starch gel was carried out at pH 8.4 using the buffer system described in the text. The anode is at the bottom of the photograph.

METHODS AND MATERIALS

Hemoglobin A was isolated from human blood by a modification of the method of Drabkin (5). Carbonmonoxyhemoglobin was separated into its subunits by treatment with p-mercuribenzoate (PMB) according to the procedure of Bucci and Fronticelli (6). After standing in the cold for 3-5 hours, the PMB-treated hemoglobin was dialyzed overnight against 10 mM phosphate buffer, pH 6.0, and the subunits were separated from each other by starch block electrophoresis using 0.06 M sodium phosphate buffer, pH 7.0. Electrophoresis was carried out on plastic plates (20 cm x 21 cm) on which 80 grams of starch containing 1% PMB had been layered (7,8). The PMB-containing alpha subunits (αPMB) moved toward the cathode and were cut out of the starch block. The protein was eluted from the starch and the PMB was removed from the alpha subunits by emulsification with cold 1-dodecanethiol according to the method of De Renzo, et al. (9).

Apo-α-subunits were prepared by acid acetone treatment (10). Immediately after heme removal, the apo-α-chains were dissolved and dialyzed against a large volume of 10 mM phosphate buffer, pH 7.0. The substantial precipitate

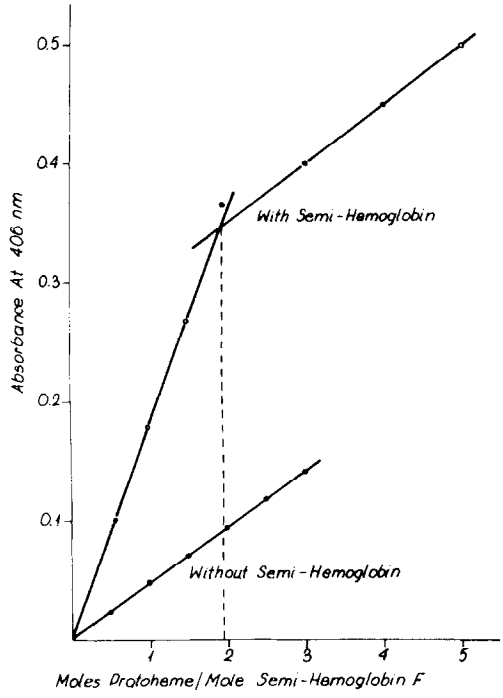

Figure 2: A spectrophotometric titration of the $\alpha_2^{\circ}\gamma_2$ semihemoglobin with protoheme. The titration was carried out on 0.1 M phosphate buffer, pH 7.0.

obtained after overnight dialysis was discarded and the soluble apo-α-subunits were placed in 0.1 M phosphate buffer, pH 7.0, and titrated with ferri-protoheme by following the absorbance change at 406 nm. The protoheme concentration was measured by pyridine hemochromogen determination (11). From the titration it was determined how much native, heme-containing gamma subunit was necessary to add to a certain volume of apo-α-subunits in order to prepare the semihemoglobin.

Fetal hemoglobin was prepared from cord blood according to the method of Stöffler, et al. (12). Gamma subunits were prepared from unfractionated lysates of fetal blood by the method of Noble (13). After treatment of the cord blood hemolysate with PMB, the pH was adjusted to 4.3 and the reaction mixture allowed to stand overnight. A single band was seen on starch block electrophoresis having a mobility indicative of that of gamma subunits. After elution of this band from the starch, the PMB was removed as described above.

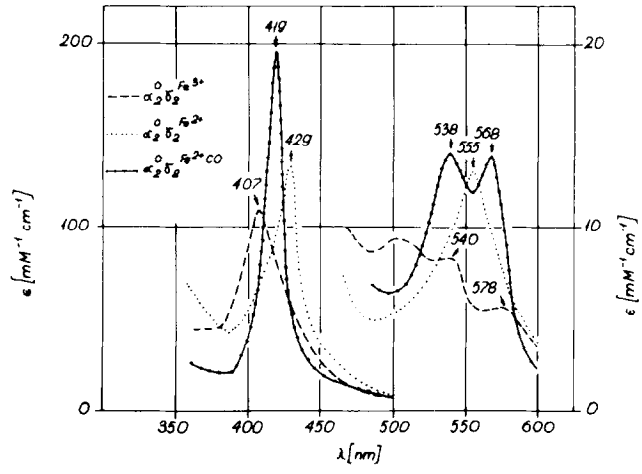

Figure 3: Light absorption spectra of the $\alpha_2^{\circ}\gamma_2$ semihemoglobin in its met, deoxy, and carbonmonoxy forms. The spectra were obtained in 0.1 M phosphate buffer, pH 7.0.

The gamma subunits were obtained in the ferric form and were titrated to the ferrous form with sodium dithionite. Identification of the gamma subunit was carried out by alkaline denaturation rate and UV absorption spectrum (14). The sulfhydryl content of all subunits described was determined before and after PMB removal by the method of Boyer (15).

The semihemoglobin, $\alpha_2^{\circ}\gamma_2$, was prepared by adding heme-containing gamma subunits to apo-α-subunits and allowing the mixture to stand at 4°C overnight. The semihemoglobin was purified and concentrated on CM-cellulose by elution with 0.1 M phosphate buffer, pH 7.5 (4).

Starch gel electrophoresis was carried out using the discontinuous buffer system described by Poulik (16). The oxygen-binding properties of the semihemoglobin were determined by using the sodium borohydride reduction technique of Asakura, et al. (17), in 0.2 M phosphate buffer, pH 7.0, at 23°C. No more than four different oxygen tensions were measured on an individual sample of semihemoglobin in order to be certain that no appreciable denaturation took place. The optical spectra of the semihemoglobin in 0.1 M phosphate buffer, pH 7.0, were measured on a Spectromom 202 spectrophotometer.

RESULTS AND DISCUSSION

Evidence for the formation of the $\alpha_2^{\circ}\gamma_2$ semihemoglobin is shown in the

starch gel electrophoresis pattern in Fig. 1. The semihemoglobin (B) appears homogeneous and has a similar mobility to that of hemoglobin F (C) and a very different mobility from that of isolated gamma subunits (A). Further evidence for the semihemoglobin formation is supplied by the fact that isolated gamma subunits are not absorbed on CM-cellulose, pH 6.0; yet after mixing apo-α-subunits with heme-containing γ-subunits, most of the heme-containing material is absorbed to CM-cellulose and must be eluted off at higher pH values.

Titration of the semihemoglobin with protoheme as followed spectrophotometrically at 406 nm is shown in Fig. 2. The titration clearly shows the binding of two moles of protoheme per mole of semihemoglobin, thereby confirming the nature of the combination product. The absorption spectra of the met, deoxy, and carbonmonoxy forms of the semihemoglobin are seen in Fig. 3, and are very similar to those obtained with native hemoglobin F.

The Hill plot obtained from oxygen equilibrium studies carried out on the semihemoglobin, hemoglobin A, hemoglobin F, and isolated gamma subunits is shown in Fig. 4. The lines are drawn from least-square approximations of the results of several experiments and the oxygenation parameters for these lines are found in Table I. The isolated gamma subunit has a high oxygen affinity and a Hill coefficient near one. The oxygen affinity of the semihemoglobin is lower than that of the isolated subunit but not nearly so low as that of either native hemoglobin A or F. In addition, it has a Hill coefficient near unity. The fact that the tetrameric semihemoglobin shows no cooperative oxygen binding is expected because the major cooperative interaction between alpha and gamma subunits during oxygenation does not occur in the semihemoglobin. The oxygen affinity of the semihemoglobin is between that of isolated gamma subunits and that of native hemoglobin F. This result is similar to that obtained previously with other hybrid hemoglobins (4,5) and indicates that the low oxygen affinity state of native hemoglobin requires that a prosthetic group be present in each subunit of the tetramer.

It is also seen in Table I that the oxygen affinity of the semihemoglobin

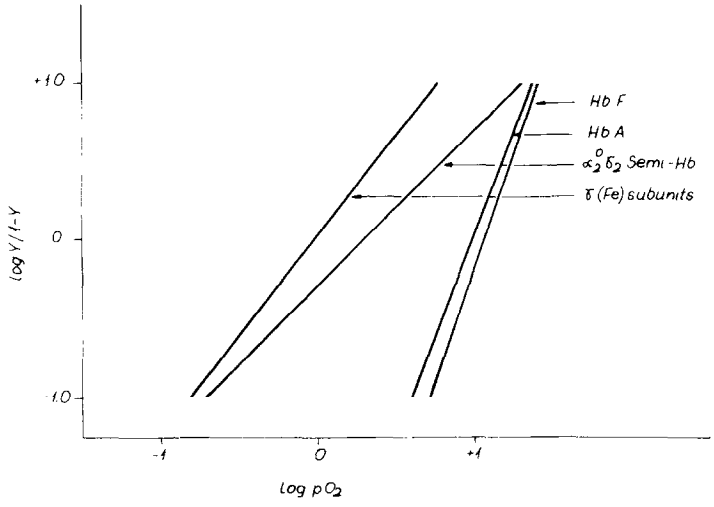

Figure 4: Hill plot of oxygen equilibria for the $\alpha_2^{\circ}\gamma_2$ semihemoglobin compared to hemoglobin A, hemoglobin F, and isolated gamma subunits. All lines were drawn from least-square approximations of the results of several oxygen equilibrium experiments. The oxygenation parameters for these lines are found in Table I. All experiments were carried out in 0.2 M phosphate buffer, pH 7.0, at 23°C.

TABLE I

Oxygenation Parameters

Sample	Log $p_{1/2}O_2$ [a]	n-value [b]
Hemoglobin A	0.98 ± 0.08 [c]	2.9 ± 0.17
Hemoglobin F	1.05 ± 0.06	3.1 ± 0.19
γ-subunit	-0.01 ± 0.04	1.3 ± 0.18
$\alpha_2^{\circ}\gamma_2$ semihemoglobin	0.31 ± 0.07	1.1 ± 0.19
β-subunit [d]	-0.04 ± 0.05	1.9 ± 0.12
$\alpha_2^{\circ}\beta_2$ semihemoglobin [d]	0.19 ± 0.06	1.08 ± 0.16

[a] $p_{1/2}O_2$ is the oxygen pressure at one-half hemoglobin saturation.

[b] n-value is the exponent in the Hill equation.

[c] ± standard error.

[d] See reference 4.

described here is somewhat lower than that of the previously described semihemoglobin containing heme in the beta subunits. In addition, the oxygen affinity of isolated beta subunits is somewhat higher than that of isolated gamma subunits. The fact that the same pattern of oxygen affinities observed in isolated subunits is maintained in the semihemoglobins offers further suggestion that a non-equivalence of hemoglobin subunits exists in the tetrameric configuration. It seems clear from the study of semihemoglobins that the α-β or α-γ interactions in the hemoglobin tetramer play an important role in decreasing the oxygen affinity of the individual subunits so that they function in the normal physiological range of oxygen concentrations. In addition, studies with semihemoglobins show that a prosthetic group in each subunit is a necessary requirement for this lowered oxygen affinity.

REFERENCES

1. Winterhalter, K.H., and Dermalean, D.A. (1967) Biochemistry 6, 3136.
2. Winterhalter, K.H., Amiconi, G., and Antonini, E. (1968) Biochemistry 7, 2228.
3. Cassoly, R., and Banerjee, R. (1971) Eur. J. Biochem. 19, 514.
4. Waterman, M.R., Gondko, R., and Yonetani, T. (1971) Arch. Biochem. Biophys. 145, 448.
5. Waterman, M.R., and Yonetani, T. (1970) J. Biol. Chem. 245, 5847.
6. Bucci, E., and Fronticelli, C. (1965) J. Biol. Chem. 240, PC 551.
7. Neer, E.J., and Guidotti, G. (1970) J. Biol. Chem. 245, 570.
8. Rosemeyer, M.A., and Huehns, E.R. (1967) J. Mol. Biol. 25, 253.
9. De Renzo, E.C., Ioppola, C., Amiconi, G., Antonini, E., and Wyman, J. (1967) J. Biol. Chem. 242, 4850.
10. Matsuda, G., Schroeder, W.A., and Martin, N. (1961) Biochim. Biophys. Acta 54, 583.
11. Paul, K.G., Theorell, H., and Akeson, A. (1953) Acta Chem. Scand. 7, 1284.
12. Stöffler, G., Flohé, L., and Tischendorf, F.W. (1966) Nature 211, 768.
13. Noble, R.W. (1971) J. Biol. Chem. 246, 2972.
14. Huehns, E.R., Dance, N., Shooter, E.M., Beaven, G.H., and Gratzer, W.B. (1962) J. Mol. Biol. 4, 329.
15. Boyer, P.D. (1954) J. Am. Chem. Soc. 76, 4331.
16. Poulik, M.D. (1957) Nature 180, 1477.
17. Asakura, T., Kawai, Y., Yoneyama, Y., and Yoshikawa, H. (1964) Anal. Biochem. 7, 393.