Absorption Spectra and Reaction with Haptoglobin of Hemoglobin (α-NO, β-Unliganded)

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SUMMARY

Human hemoglobin (αNOβ), in which α chains were liganded with nitric oxide and β chains were unliganded, showed a characteristic spectrum in the Soret region. Its absorption was significantly reduced compared with the averaged absorption of α-NO and β. On the other hand, a complex of (αNOβ) with haptoglobin did not show such a characteristic spectrum. It was concluded, therefore, that there are some subunit interactions in (αNOβ) and that they are lost when (αNOβ) is bound to haptoglobin. Reaction of (αNOβ) with haptoglobin was followed spectrophotometrically, and it was found that the rate of reaction of (αNOβ) with haptoglobin was markedly different from that of fully liganded or fully unliganded hemoglobin with haptoglobin. (αNOβ) seems to be in a different conformational state from both fully liganded or fully unliganded hemoglobin.

Haptoglobin (Hap) is a plasma glycoprotein, which binds fully liganded hemoglobin in an essentially irreversible reaction to give a very stable complex (1), but does not bind unliganded hemoglobin (2, 3). According to Gibson and his group (4, 5), dissociation of tetrametric hemoglobin to the dimer is a prerequisite for Hap binding, and the apparent inability of Hap to bind unliganded hemoglobin is due to the very small dissociation constant of unliganded hemoglobin at neutral pH.

It seems of interest to investigate the reaction with Hap of mixed hybrid hemoglobins, in which one pair of chains is kept locked in the liganded form and the other pair is unliganded, because the hybrids are considered to possess the properties of the reaction intermediates with ligands and presumably to take the structure of the intermediate state. One of the types of the hybrids often studied is the "NO-hybrid" in which one pair of chains is liganded with nitric oxide (NO). This hybrid is relatively stable because NO dissociates with extreme slowness (6).

In this paper we report the characteristic absorption spectra of hemoglobin, (αNOβ), in which the α chains are liganded with NO and the β chains are unliganded, and, taking advantage of this absorption spectra, the reaction of (αNOβ) with Hap.

MATERIALS AND METHODS

The "isolated αII chains" were prepared from human hemoglobin according to the method of Bucci and Fronticelli (7) with a slight modification. The NO-derivative of the α chain, 1 ml of solution containing about 3 mg of protein in 0.1 m sodium phosphate buffer, pH 7.0, was converted to the deoxynated form in N2. Then a mixture of 5% NO in N2 was introduced anerobically and, after gentle shaking, the solution was quickly applied to a column (1.5 × 27 cm) of Sephadex G-25 equilibrated with 0.1 m sodium phosphate buffer, pH 7.0, to remove excess NO and any oxidation products of NO present in the solution. The top of the column was filled previously with 1 ml of sodium dithionite solution (2 mg per ml). All the experiments were performed within a few hours after preparation.

Purification of Hap 2-2 from human sera was carried out according to the method of Black et al. (8) except that the final Sephadex G-200 column was eluted with 0.1 m sodium phosphate buffer, pH 7.0, instead of 0.05 m ammonium acetate, pH 5.5. The eluate containing Hap 2-2 was stored at −15°C. If the eluate was slightly turbid, it was cleared by treatment with ammonium sulfate at 35% saturation at 0°C. To Hap 2-2 thus obtained, an excess amount of human oxyhemoglobin was added and the mixture was analyzed by acrylamide gel electrophoresis (9). One half of the gel was stained with Amido black 10B and the other half was stained with o-Dianisidine (to 100 ml of a solution of o-Dianisidine in 0.5% acetic acid, 0.2 ml of 30% H2O2 was added immediately before use). No protein band other than the Dianisidine-positive one was found, indicating the considerable purity of Hap 2-2. The reaction of fully liganded hemoglobin, O2- and NO-hemoglobin, with Hap 2-2 was checked by measuring the quenching of Hap fluorescence associated with complex formation (10), and it was confirmed that the rate of the reaction is roughly the same as that of O2-
hemoglobin with Hap 1-1 reported by Nagel and Gibson (10), although the reaction was so fast that we would not follow it exactly. It was also confirmed that Hap 2-2 is never bound to fully unliganded hemoglobin within an hour. The absorption spectrum of the Hap 2-2 in the Soret region was measured and a very small absorption at 400 to 440 nm was observed, probably due to a small contamination with the hemoglobin-Hap complex present in the sera. This contribution to the spectrum was subtracted in all the experiments.

RESULTS AND DISCUSSION

The complex, (αNOβ)-Hap, was prepared as follows. To the mixture of an equivalent amount of α-NO and β-O2, Hap was added to make a solution of a final concentration of 4.7 μM in heme and 154 μg per ml in Hap, respectively. After a few minutes, the mixture was deoxygenated with sodium dithionite. The Soret band absorption spectra of (αNOβ) and (αNOβ)-Hap are shown in Fig. 1. For at least 30 min both spectra did not change. Therefore, dissociation of NO from α-chains during that time can be neglected. A clear difference between the spectra of (αNOβ) and (αNOβ)-Hap was found in the region of 410 to 430 nm. Difference spectra between (αNOβ) or (αNOβ)-Hap and their unmixed constituents were recorded using the tandem cell system essentially as described by Herskovitz and Laskowski (11). As shown in Fig. 2, the spectra of (αNOβ)-Hap were found to be nearly equal to an average of those of α-NO and β, whereas the spectra of (αNOβ) were not a simple sum of those of their components. This indicated clearly that in (αNOβ) there are some subunit interactions between α and β chains. A similar difference was observed between unliganded hemoglobin and the unliganded hemoglobin-Hap complex. As proposed previously by Chiancone et al. (3), the spectra of the unliganded hemoglobin-Hap complex are an average of those of unliganded α and β chains, whereas the spectra of unliganded hemoglobin are not. However, the striking difference between unliganded hemoglobin and the (αNOβ) hybrid is that the absorbance at 410 to 435 nm of unliganded hemoglobin is larger than the average absorbance of its constituent chains, whereas the absorbance at the same region of (αNOβ) is always smaller than the averaged absorbance. There might be characteristic subunit interactions in (αNOβ).

The spectral difference between (αNOβ) and (αNOβ)-Hap enables us to investigate the reaction of (αNOβ) with Hap, because the extinction at 417 nm of (αNOβ) increases as it is bound to Hap. Fig. 3 shows results of the kinetic experiments in which (αNOβ) is bound to various concentrations of Hap. A half-time for the combination of (αNOβ) and Hap was found to be approximately 2 min at all concentrations of Hap tested. It should be mentioned that the method using a quenching of Hap fluorescence (10) was not adequate in this case because the reaction was relatively slow and dithionite interfered strongly with the fluorescence. In contrast to (αNOβ), unliganded hemoglobin is hardly bound to Hap and fully liganded hemoglobin is bound to Hap with a half-time of a few seconds (10). Thus, the rate of reaction of (αNOβ) with Hap is in between those of the fully liganded and the fully unliganded hemoglobins with Hap. This finding supports the idea that the conformational state of (αNOβ) is different from that of fully liganded or

Fig. 1. Absorption spectra of (αNOβ) and (αNOβ)-Hap. —, spectrum of (αNOβ); concentration was 4.7 μM in heme; — — —, spectrum of (αNOβ)-Hap; concentration was 4.7 μM in heme and 154 μg per ml of Hap 2-2; further addition of Hap 2-2 did not alter the spectrum. (αNOβ)-Hap was prepared as described in the text. All samples were in 0.1 m sodium phosphate buffer, pH 7.0, with 1.5 mg per ml of sodium dithionite.

Fig. 2. Difference spectra of (αNOβ) and (αNOβ)-Hap against their components. —, (αNOβ) minus α-NO and β; — — —, (αNOβ)-Hap minus α-NO, β, and Hap. Final concentration of (αNOβ) and Hap 2-2 were the same as in Fig. 1. All samples were in 0.1 m sodium phosphate buffer, pH 7.0, with 1.5 mg per ml of sodium dithionite.

Fig. 3. Reaction of (αNOβ) with Hap 2-2 of various concentrations. The absorbance of (αNOβ) at 417 nm was followed after mixing with Hap 2-2. Temperature was kept constant at 23°. Final concentration of (αNOβ) was 4.7 μM in heme and the experiments were done in 0.1 m sodium phosphate buffer, pH 7.0, with 1.5 mg per ml of sodium dithionite.
fully unliganded hemoglobin, since the dissociation constant of the tetrameric (αNOβ) to dimers seems to have an intermediate value between those of the fully liganded and the fully unliganded hemoglobin, as judged by the rate of reaction of (αNOβ) with Hap.

Another type of hybrid, (αβNO), was also studied. However, its absorption spectra were not stable and a definite spectral difference between (αβNO) and (αNO)-Hap was not found.

There are some reports on valency hybrid hemoglobins, in which hemes of one pair of chains are maintained in the ferric form and those of the partner chains are maintained in the ferrous form and are free to be oxygenated or deoxygenated. The value of $n$ in the Hill equation for (α-cyanmet, β) was somewhat larger than unity (12, 13), and the reactivity of the $\beta NO$ sulphydryl groups of the hybrid with $p$-mercuribenzoate was intermediate between those of the fully liganded and fully unliganded hemoglobins (13). On the contrary, the value of $n$ of another kind of hybrid, (α,β-cyanmet), is unity and the reactivity of the sulphydryl groups of the hybrid with $p$-mercuribenzoate is close to that of fully liganded hemoglobin (13). Furthermore, Henry and Banerjee have studied NO-hybrids of hemoglobin by electron paramagnetic resonance spectroscopy (14). The spectra of (αNOβ) depended on the spin state of the heme carried by the β chain, whereas the spectra of (αβNO) were always identical to those of the isolated βNO subunits. Studies on electron paramagnetic resonance spectra of valency hybrid hemoglobins led them to the similar conclusions (15). These observations and the experimental results presented here confirm the view that there is a conformational difference between hemoglobin (α-liganded, β-unliganded) and hemoglobin (α-unliganded, β-liganded), and hemoglobin (α-liganded, β-unliganded) has subunit-subunit interactions and its conformational state is different from those of both the fully liganded and the fully unliganded hemoglobins. The nature of subunit interactions and the quaternary structure of this hybrid hemoglobin will be studied further.

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