OBSERVATION OF ALLOSTERIC TRANSITION IN HEMOGLOBIN

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Summary: Two conclusions have been drawn from NMR studies of mixed state hemoglobins. First the α and β subunits in hemoglobin are not equivalent in their conformational properties. Second the mixed state hemoglobin $(\alpha^{III}CN\ \beta^{II})_2$ can take two different quaternary structures without changing the degree of ligation. One of the two structures is similar to that of deoxyhemoglobin and the other to that of oxyhemoglobin.

INTRODUCTION

It has previously been shown (1-4) that heme proteins in paramagnetic states have a number of well resolved NMR peaks shifted to higher and lower fields. These peaks are shifted by interactions with the paramagnetic electrons and their positions are sensitive indicators of the structure and electronic properties of the heme and its environment.

Many recent experiments relating to the oxygenation of hemoglobin have been performed on mixed state hemoglobins where one type of subunit, either α or β, is kept in the ferric state and complexed with cyanide, while the other type is in the normal ferrous state and can be alternately oxygenated or deoxygenated (4-10).

In this brief report we discuss our recent high resolution NMR experiments on mixed state hemoglobins, presented separately in detail (11), which show that for the same number of ligands, i.e. two, mixed state hemoglobin can exist in two different states characterized by two different NMR spectra. The equilibrium between these two states can be shifted by the addition of 2,3-diphosphoglycerate (DPG), which is known to stabilize

deoxyhemoglobin (12,13). This suggests that the two different states are closely related to the oxy and deoxy quaternary structures (14).

MATERIALS AND METHODS

The α and β subunits were separated by p-chloromercuribenzoate reaction (15) and subsequent CMC chromatography. Regeneration of SH group in the α subunits was done with mercaptoethanol treatment (16) on CMC. N-acetyl pennicillamine (17) was used for the β subunits. Oxidation of the isolated subunits to the cyanoferric state was carried out as described by Tyuma, Benesch and Benesch (18). Equimolar mixtures of $\alpha^{III}CN$ and $\beta^{II}O_2$ or $\alpha^{II}O_2$ and $\beta^{III}CN$ were further chromatographed on CMC.

A Varian HR 220 NMR spectrometer was used for the experiments. The line positions are described by the separation in parts per million, ppm, from the position of DSS, 2,2-dimethyl-2-silapentane-5-sulfonate, with downfield shifts assigned negative numbers. The spectra were obtained by accumulating the signal in a Fabritek 1062 computer for 1 to 6 hours.

RESULTS

In mixed state cyano plus oxyhemoglobins the paramagnetic cyanoferric hemes give temperature dependent resonances at fields above and below the normal diamagnetic region, 0 to -10 ppm from DSS. Figure 1 shows the spectra from -10 to -25 ppm of the $\alpha^{III}CN$ and $\beta^{III}CN$ subunits in the two mixed state hemoglobins $\alpha^{III}CN\ \beta^{II}O_2$ and $\alpha^{II}O_2\beta^{III}CN$. These results will be discussed in more detail elsewhere (19). Two points should be noted. As seen in Fig. 1, α and β subunits in the cyanoferric forms give slightly different spectra and they can be distinguished in the spectrum of $Hb^{III}CN$ which is a superposition of the spectra of α and β subunits in the fully ligated mixed state

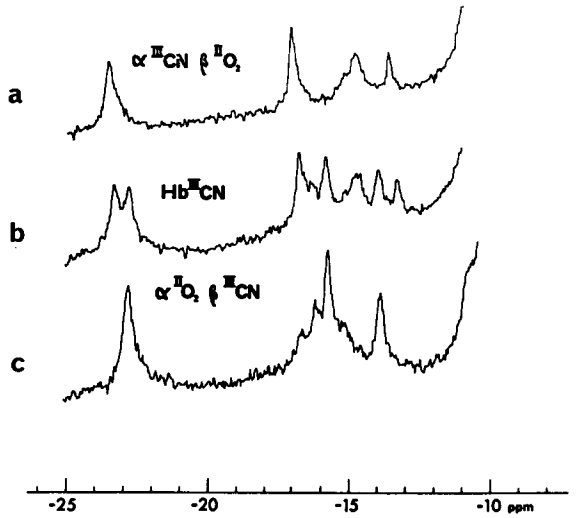

Fig. 1. Comparison of NMR spectra of mixed state hemoglobins and cyanoferric hemoglobin. (a) $(\alpha^{III}CN\ \beta^{II}O_2)_2$; (b) $Hb^{III}CN$; (c) $(\alpha^{II}O_2\beta^{III}CN)_2$ in pH 6.6 phosphate-D_2O buffer at 20°C.

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hemoglobins. The other point shown in separate experiments (19) is that the spectra in Fig. 1 were very sensitive to pH in phosphate buffer and the changes of line positions were titratable with pH in the physiological pH region.

The deoxygenation of $(\alpha^{II}O_2\beta^{III}CN)_2$ in phosphate buffer at pH 7.2 in D_2O did not produce any changes in the paramagnetically shifted resonance lines of the $\beta^{III}CN$ subunits, neither in the low field region of -10 to -25 ppm, nor in the high field region of +2 to +6 ppm. On the other hand, under certain conditions definite changes in the NMR spectrum of $(\alpha^{III}CN\ \beta^{II}O_2)_2$ were observed upon deoxygenation. When $(\alpha^{III}CN\ \beta^{II}O_2)_2$ in phosphate buffer at pH 7.1 was deoxygenated, all the paramagnetically shifted lines of $\alpha^{III}CN$ subunits in the observable region of resonance (-10 to -25 and +2 to +6 ppm) showed substantial shifts in their positions. These changes in the upfield region are shown in Fig. 2, where the line at

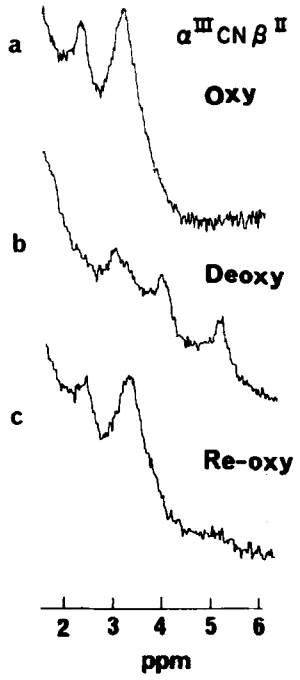

Fig. 2. NMR spectra in high field region. (a) $(\alpha^{III}CN\ \beta^{II}O_2)_2$; (b) $(\alpha^{III}CN\ \beta^{II})_2$; (c) sample (b) re-oxygenated in phosphate buffer at pH 7.1, 15°C.

+2.4 ppm comes from the oxygenated β^{II} subunit and disappears upon deoxygenation, while the other lines come from the $\alpha^{III}CN$ subunit.

When similar experiments were carried out at higher pH's (7.6 instead of 7.1) with Bis Tris buffer, instead of phosphate, these large spectral changes upon deoxygenation were not observed (Fig. 3(d-f)). However there were very small shifts of the peaks in the upfield region as can be seen in Fig. 3(d-f) while no appreciable change occurred in the downfield region. However, when a two-fold excess of DPG per tetramer of $(\alpha^{III}CN\ \beta^{II}O_2)_2$ was added, the large spectral changes observed, Fig. 3(a-c), were very similar to the changes observed upon deoxygenation without DPG at pH 7.1 in phosphate buffer (see

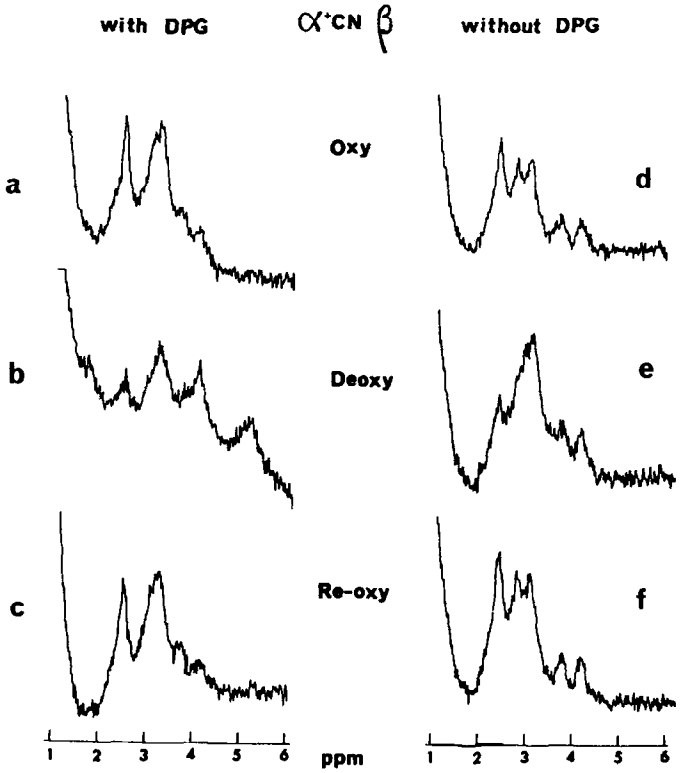

Fig. 3 Effect of DPG on NMR spectra of $(\alpha^{III}CN\ \beta^{II})_2$ mixed state hemoglobin in high field region. (a) $(\alpha^{III}CN\ \beta^{II}O_2)_2$ with DPG; (b) $(\alpha^{III}CN\ \beta^{II})_2$ with DPG; (c) sample (b) re-oxygenated; (d) $(\alpha^{III}CN\ \beta^{II}O_2)_2$ without DPG; (e) $(\alpha^{III}CN\ \beta^{II})_2$ without DPG; (f) sample (e) re-oxygenated in 0.2 M Bis Tris buffer pH 7.6 at 15°C. The ratio of DPG to hemoglobin tetramer was 2.

Fig. 2). Furthermore the oxygen affinity in the pH 7.6 system without DPG was substantially higher than in the system with DPG as determined qualitatively by the ease of deoxygenation.

We suggested that the altered spectrum arises from molecules having a quaternary structure which is stabilized by DPG, and therefore is very similar to that of deoxyhemoglobin. At the intermediate pH 7.3, and in phosphate buffer but without DPG, it was possible to observe the superposition of

the two spectra with approximately equal intensities, indicating that the two states responsible for these spectra were in equilibrium. Furthermore because no additional broadening of the NMR lines was observed in the pH 7.3 mixture, we estimate that the lifetime of these states must be longer than 6 msec.

A comparison of these NMR spectral changes with the ligand binding properties, both equilibrium and kinetic, reported by others (9,10) suggests that they may be related. This is being investigated.

In light of the present experiments and of many other previous studies, the most likely mechanism for the cooperative oxygen binding of hemoglobin seems to be a "modified allosteric transition model". Since the hemes and the heme-ligand bond are not responsible for the 2 to 3 kcal/mol of interaction energy (4), the large difference in oxygen affinity between the two forms of hemoglobin has to come from the protein part and especially from the interfaces of the subunits. It is likely to be the case that ligand induced tertiary structural changes extend to the interfaces of the subunits where those changes are more strongly stabilized in one quaternary structure than in the other, and this leads to differences in oxygen affinity between the two structures. In this modified allosteric model, both tertiary (20) and quaternary (21) structures have important roles in the cooperative oxygen binding.

REFERENCES

1. Kowalsky, A., Biochemistry 4, 2382 (1965).
2. Wüthrich, K., Shulman, R. G. and Peisach, J., Proc. Nat. Acad. Sci. (U.S.) 60, 373 (1968).
3. Kurland, R. J., Davis, D. G. and Ho, C. J., Amer. Chem. Soc. 90, 2700 (1968).
4. Shulman, R. G., Ogawa, S., Wüthrich, K., Yamane, T., Peisach, J. and Blumberg, W. E., Science 165, 251 (1969).
5. Ogawa, S., McConnell, H. M. and Horwitz, A., Proc. Nat. Acad. Sci. (U.S.) 61, 401 (1968).
6. Antonini, E. and Brunori, M., J. Biol. Chem. 244, 3909 (1969).

7. Enoki, Y. and Tomita, S., J. Mol. Biol. 32, 121 (1968).
8. Banerjee, R. and Cossoly, R., J. Mol. Biol. 42, 251 (1969).
9. Brunori, M., Amiconi, G., Antonini, E., Wyman, J. and Winterhalter, K. H., J. Mol. Biol. 49, 461 (1970).
10. Haber, J. C. and Koshland, D. E. (to be published).
11. Ogawa, S. and Shulman, R. G., J. Mol. Biol. (to be published).
12. Benesch, R. and Benesch, R. E., Biochem. Biophys. Res. Commun. 26, 162 (1967).
13. Chanutin, A. and Curnish, R. R., Arch. Biochem. Biophys. 121, 96 (1967).
14. Perutz, M. F., Proc. Roy. Soc. (London) B173, 113 (1969).
15. Bucci, E. and Fronticelli, C., J. Biol. Chem. 240, 551 (1965).
16. Geracci, G., Parkhurst, L. J. and Gibson, Q. H., J. Biol. Chem. 244, 4664 (1969).
17. Benesch, R., Benesch, R. E. and Enoki, Y., Proc. Nat. Acad. Sci. (U.S.) 61, 1102 (1968).
18. Tyuma, I., Benesch, R. E. and Benesch, R., Biochemistry 5, 2957 (1966).
19. Ogawa, S., Shulman, R. G., Yamane, T. and Fujiwara, M. (to be published).
20. Koshland, D. E., Nemethy, G. and Filmer, D., Biochemistry 5, 365 (1966).
21. Monod, J., Wyman, J. and Changeux, J. P., J. Mol. Biol. 12, 88 (1965).