INFLUENCE OF CHEMICAL MODIFICATIONS OF THE REACTIVE SH GROUPS ON THE PROTON BINDING BEHAVIOUR OF HUMAN AND HORSE HEMOGLOBIN

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SUMMARY

From a proton-binding study of human HbCO with the reactive $\beta 93$ SH groups substituted by iodoacetamide, we found that in this derivative two imidazole groups more appear to be titratable than in HbCO. In order to investigate the specificity of iodoacetamide in this respect, we have studied the influence of two other commonly used SH reagents, *viz.* *N*-ethylmaleimide and *p*-mercuribenzoate.

1. Reaction of *N*-ethylmaleimide and *p*-mercuribenzoate with human HbCO does not lead to the unmasking of two imidazole groups.

2. Upon blocking the SH groups with *p*-mercuribenzoate, two groups from the neutral region change their pK from 7 to 8.

3. Horse HbCO behaves analogously to human HbCO. The pK_{SH} however seems to be higher than in human HbCO.

In a recent paper¹ we presented a proton-binding study of human hemoglobin and some of its derivatives. Comparing the results for HbCO and HbCO-IAA*, it appeared that upon iodoacetamide substitution of the reactive $\beta 93$ SH groups two imidazole groups were unmasked. This result was unexpected in view of the fact that analogous substitution by *N*-ethylmaleimide does not show detectable structural differences between substituted and unmodified hemoglobin². Therefore we have studied the influence of two other SH blocking reagents, *viz.* *N*-ethylmaleimide and *p*-mercuribenzoate, on the proton-binding behaviour of HbCO.

HbCO-NEM was prepared in a similar way to that described³ for the preparation of HbCO-IAA. HbCO-PMB was prepared as follows: A solution of *p*-mercuribenzoate, prepared as described by BUCCI AND FRONTICELLI⁴, was added to an unbuffered 4% solution of HbCO. After the addition the pH was about 7. The mixture was allowed to react overnight at 4° and then dialyzed against distilled water. By using about 3 moles *p*-mercuribenzoate per mole HbCO, no subunits are

* Abbreviations: HbCO-IAA, HbCO-NEM and HbCO-PMB, carbon monoxide hemoglobin after reaction with iodoacetamide, *N*-ethylmaleimide and *p*-mercuribenzoate respectively.

Fig. 1. Differential titration curves of human HbCO (— — —), HbCO-NEM (○) and HbCO-PMB (●). Protein concn. about 20 mg/ml; $I = 0.1$; temperature 25°.

produced and only the SH groups at position $\beta 93$ are substituted⁵. The titration curves were measured and analysed as described earlier¹. For the meaning of the symbols used we refer to that paper.

Fig. 1 shows the differential titration curves of these modified proteins together with that of the untreated HbCO. The position of the second peak in the differential titration curve of HbCO-NEM remained unaltered at $Z_H = -10$, as in the case of HbCO-IAA¹. This means that the SH groups were not titrated between the two peaks. Only the height of the right peak changed from about 0.30 in the case of HbCO to 0.32 for HbCO-NEM; the same change of 0.02 in $-\Delta pH/\Delta Z_H$ was found upon iodoacetamide substitution, and so far this confirms our earlier reported value of 9.9 for pK_{SH} . In contrast to HbCO-IAA the distance between the two peaks was not increased but even somewhat diminished. Therefore the liberation of two groups as observed in HbCO-IAA probably was a specific effect of iodoacetamide. The decrease in peak-to-peak distance in HbCO-NEM relative to HbCO might be caused by an additional reaction of *N*-ethylmaleimide with the α -amino groups of the α -chains⁶. This would also explain the fact that the isoionic pH of HbCO-NEM was somewhat lower as compared with that of HbCO (7.17 and 7.25 respectively) because the pK of the α -amino group of the α -chain is near 6.7 in human HbCO⁷. The fact that Z_H was not affected after binding of *N*-ethylmaleimide (the calculated shift based on $pK_{SH} = 9.9$ is about 0.1, which is within the experimental accuracy) proves that the possible formation of two extra carboxyl groups due to the hydrolysis of

the succinimide ring, as proposed by BENESCH AND BENESCH⁸, is not very likely because this phenomenon should have shifted the right peak to -12 .

In Fig. 1 also the differential titration curve of HbCO-PMB is shown. Due to *p*-mercuribenzoate substitution two carboxyl groups are introduced in the hemoglobin. This explains the fact that the titration curve is displaced two charge units to the right. The peak-to-peak distance is however the same as in HbCO, indicating that *p*-mercuribenzoate substitution also does not produce an unmasking of imidazole groups. There is another remarkable difference with HbCO-NEM: whereas the experimental value of $-\Delta\text{pH}/\Delta\bar{Z}_H$ at Z_H for HbCO-NEM was about 0.32, this value was only 0.27 for HbCO-PMB. This indicates that upon substitution with *p*-mercuribenzoate either some groups from the neutral region (*i.e.* the region between the two peaks) have obtained a higher *pK* or that some groups from the basic region have obtained a lower *pK*. The former explanation is favoured by the following reasoning: At $\bar{Z}_H = 0$ $-\Delta\text{pH}/\Delta\bar{Z}_H$ was near 0.1 and therefore, in view of the presence of two extra carboxyl groups in HbCO-PMB and because the isoionic pH of HbCO was 7.25, one should expect the isoionic point of this protein 2×0.1 lower, (near pH 7.05) while experimentally 7.20 was found. We calculated that the abnormal peak height and isoionic pH of HbCO-PMB corresponded with a shift in *pK* of two groups from about 7 in HbCO to 8 in HbCO-PMB.

A possible explanation for this observation could be the following: in Hb there seems to exist a hydrogen bond between His HC3(146) β and the carboxyl group of Asp FG1(94) β^2 . It is possible that a similar type of bond exists in HbCO-PMB, but this time between the carboxyl group of *p*-mercuribenzoate coupled at Cys F9(93) β and the same His residue.

Similar experiments were performed on horse hemoglobin⁹. Here it was found that *pK*_{SH} in horse HbCO was higher than in human HbCO because substitution with iodoacetamide did not influence the height of the peak at Z_H . After blocking with iodoacetamide, *N*-ethylmaleimide and *p*-mercuribenzoate the same side reactions were observed as in the case of human hemoglobin.

The results described here are in our opinion an indication that changes in the functional properties of hemoglobins upon substitution might be related to changes in the tertiary structure of the molecule or other side effects.

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