ON THE DISSOCIATION OF HUMAN AND PIG DEOXYHEMOGLOBINS INTO SUBUNITS

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1. Introduction

Recent experiments have shown that unliganded human hemoglobin tetramers are considerably more stable than liganded tetramers. The tetramer–dimer dissociation constant ($K_{4,2}$) for human deoxyhemoglobin (HbA) is at least about two–three orders of magnitude less than that for HbO_2 [1–3]. In all known cases the Hb dissociation into dimers is accompanied by the loss of cooperativity in ligand binding. Noncooperative dimers are produced upon a reversible dissociation of native HbA in the pH range of 10 to 11 [1–3] or they occur at neutral pH in the prescence of 2 M NaI at very low Hb concentrations ($\approx 10^{-6}$ M) [4]. Stable deoxy dimers were obtained in the presence of 0.9 M $MgCl_2$ and it was shown that they were $\alpha_1\beta_1$ dimers as in the case of liganded forms, i.e. the dissociation occurred across the interface $\alpha_1\beta_2$ [5]. Indirect data allowed to suggest that at alkaline pH HbA tetramers also were dissociated into dimers $\alpha_1\beta_1$ [1–3].

We have tried to obtain more data about the nature of noncooperative subunits occurring upon the alkaline dissociation of HbA. The present paper deals with the comparative study of the dissociation of HbA, pig deoxyhemoglobin (Hb_{pig}) and their chemically modified forms obtained after the reaction of these proteins with *p*-chloromercuribenzoate (*p*CMB). It will be shown that when two *p*CMB molecules are bound to the reactive sulfhydryl groups of Cys 93 β situated near the contacts $\alpha_1\beta_2$ [6] the dissociation of tetramers into noncooperative dimers occurs more easily, i.e. the dimers formed are really $\alpha_1\beta_1$ and not $\alpha_1\beta_2$.

Preliminary data concerning the dissociation of HbA completely reacted with *p*CMB are given.

2. Materials and methods

Human and pig hemoglobins were prepared according to the method of Rossi-Fanelli et al. [7]. The *p*CMB-treated hemoglobin was prepared in the oxygenated form according to the method of Rosemeyer and Huehns [8]. The reactions were performed at pH 6.5–6.7 at 10° in the presence of 0.2 M sodium chloride. The product of incomplete reaction of HbA with *p*CMB which has only two modified sulfhydryl groups of Cys 93 β (HbA^{2pCMB}) was obtained at molar ratio of reagent to protein 4:1 or 2:1. The reaction proceeded not more than 3 min and then the samples were passed through Sephadex G-25 equilibrated with 0.2 M NaCl to remove excess reagent. The results for two preparations were identical. The product of complete reaction of *p*CMB with six sulfhydryl groups of HbA (HbA^{6pCMB}) was prepared by the addition of a 10-fold excess of *p*CMB to HbO_2. The reaction mixture was allowed to stand about 20 hr at 10°, then it was filtered and passed through Sephadex G-25 equilibrated with 0.1 M NaCl. Pig hemoglobin was modified in a similar way.

*p*CMB (Chemarol) was purified by the method of Boyer [9].

Hemoglobin concentration was determined spectrophotometrically using the extinction coefficient for oxy form.

Dissociation of deoxyhemoglobin into subunits was followed through spectral changes by magneto-

optical rotatory dispersion (MORD) method. The fraction of dissociated molecules (f) was determined as described in previous publication [3].

The desired value of pH was obtained by mixing equal volumes of HbO_2 in 0.2 M NaCl and appropriate buffer in which a few grains of sodium dithionite (Merck) was added prior to the addition of protein. The buffers used were 0.2 M phosphate at pH 6–8 and 0.2 M glycine-phosphate NaOH at pH 8.

The cells used were 2 mm for Hb concentrations $\approx 0.5\%$ and 10 mm for Hb concentrations $\approx 0.1\%$.

All measurements were carried out at $20 \pm 0.2°$.

3. Results and discussion

We have shown earlier [10] that MORD curves are sensitive to the changes of quaternary Hb structure which are accompanied by loss of cooperative properties in ligand binding. It makes possible to use the method to study the dissociation of Hb into noncooperative subunits by measuring the changes of the value of the MORD minimum at 586 nm with respect to the value of the MORD minimum at 556 nm which remains constant if there is denatured protein [3]. Furthermore, the MORD method permits to control with high accuracy the absence of oxygenated and denatured hemoglobin in a solution under study. Hb_{pig} has the same MORD curve as HbA: the value of the minimum at 586 nm is two times greater than that at 556 nm. In the process of the dissociation into noncooperative subunits the value of the minimum at 586 nm decreases and reaches the value of the minimum at 556 nm when the dissociation is completed.

In fig. 1 the points denote the experimental values of $(1-f)$ versus pH for native HbA and Hb_{pig} (curve 1) and for HbA^{2pCMB} and Hb_{pig} (curve 2) treated by pCMB (Hb_{pig}^{pCMB}). $(1-f)$ is the fraction of Hb molecules in tetrameric form. The results for Hb_{pig}^{pCMB} do not depend on the excess of pCMB in the reaction mixture (if the ratio pCMB: $Hb_{pig} > 2$) as well as on the reaction time. Curves 1 and 2 were calculated for tetramer–dimer dissociation assuming that 3.5 protons were released in the process. The curves for respective forms of HbA and Hb_{pig} coincide within experimental error. The midpoints of the transition for native and modified hemoglobins were found at pH 10.9 and 10.6, respectively.

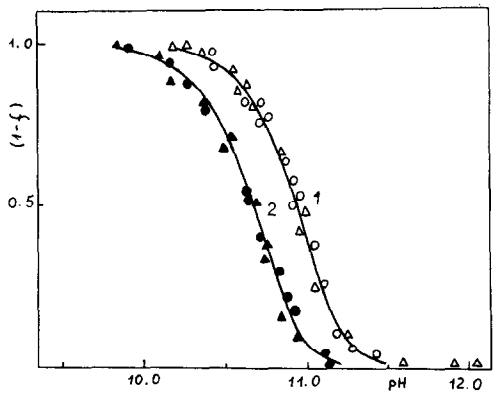

Fig. 1. Dissociation of deoxyhemoglobin at alkaline pH values. $(1-f)$ is the fraction of undissociated molecules, at 20°; concentration of Hb 0.5%; in 0.2 M phosphate-glycine buffer + 0.2 M NaCl. 0: native HbA; ▲: native Hb_{pig}; ●: HbA^{2pCMB}; ▲: Hb_{pig}^{2pCMB}

In Hb_{pig} as compared with HbA there is only one replaced amino acid residue (Pro 37 $\alpha \rightarrow$ Thr) among 20 residues taking part in the $\alpha_1\beta_2$ contacts [11]. On the other hand, among 34 amino acid residues lying at the $\alpha_1\beta_1$ interface there are 6 replacements (3 in α- and 3 in β-chain). One of them is the replacement of ionizable at alkaline pH residue Cys 112 β to the unionizable nonpolar residue Val. The dissociation of HbA into dimers $\alpha_1\beta_2$ must change the ionization state of Cys 112 β and hence influence on the position and steepness of the curve $(1-f)$ versus pH in comparison with that for Hb_{pig}. The coincidence of dissociation curves for native HbA and Hb_{pig} is consistent with the previous suggestion [1, 3] that Hb tetramers dissociate into dimers of the composition $\alpha_1\beta_1$.

The further evidence for such a mechanism of dissociation is given by data for HbA^{2pCMB} and Hb_{pig}^{pCMB}. In HbA there are six SH-groups two of which (Cys 93 β) lie at the $\alpha_1\beta_2$ interface near the contacts $\alpha_1\beta_2$ [6] and react rapidly with pCMB [14] to produce modified hemoglobin HbA^{2pCMB}. Four other SH-groups (Cys 104 α and Cys 112 β) are hidden inside the molecules and are involved in $\alpha_1\beta_1$ contacts [6]. They react with pCMB only after prolonged incubation of HbA with a large excess of pCMB [8, 15]. In Hb_{pig} there are only four SH-groups (Cys 93 β and Cys 104 α). As our experi-

ments have shown the dissociation of chemically modified Hb_{pig} does not depend on the ratio pCMB:Hb_{pig} (if pCMB:$Hb_{pig} > 2$) and on time of incubation of Hb_{pig} with pCMB. This fact is consistent with the conclusion of Rosemeyer and Huehns [8] that cysteil 104 α of Hb_{pig} does not react with pCMB at neutral pH.

The dissociation curve (2 in fig. 1) for HbA^{2pCMB} and Hb_{pig}^{pCMB} in which two sulfhydryl groups have reacted with pCMB is identical in shape but is shifted about 0.3 units of pH to lower pH value in respect to the curve for native HbA and Hb_{pig} (curve 1 in fig. 1). The shift of dissociation curve after modification of Cys 93 β which are located near $\alpha_1\beta_2$ contacts confirms the conclusion that dimers occurred upon alkaline dissociation of HbA and Hb_{pig} are $\alpha_1\beta_1$.

Since the curves $(1-f)$ versus pH for native HbA and Hb_{pig} and for modified HbA^{2pCMB} and Hb_{pig} are identical in shape and $d \log K_{4,2}/dpH = -3.5$ [3] the decrease of interaction energy at the interface of $\alpha_1\beta_2$ contacts upon the reaction of Cys 93 with pCMB is equal to 1.45 Kcal/mole (the dissociation constants $K_{4,2}$ for HbA^{2pCMB} and Hb_{pig}^{pCMB} are about one order of magnitude greater than these constants for HbA and Hb_{pig}).

It is to be noted that Hb_{pig} differs in one respect from HbA. The denaturation of HbA can be observed at pH > 11.0 [3], while in the samples of Hb_{pig} the traces of denatured protein are absent up to pH 11.9. From the spectrophotometric studies of tyrosyl titration of isolated α^{SH} and β^{SH} chains of HbA [17] it can be concluded that the chains become unstable at pH near 10.4. Therefore the pH value of the beginning of the denaturation process will give us an information about the alkaline stability of $\alpha_1\beta_1$ contacts in the dimer. If isolated α- and β-chains of Hb_{pig} become denatured at pH < 11.9 the higher stability to denaturation of Hb_{pig} demonstrates the higher stability of interchain contacts in $\alpha_1\beta_1$ dimers of Hb_{pig} as compared with the contacts in $\alpha_1\beta_1$ dimers of HbA.

In fig. 2 the preliminary data for HbA^{6pCMB} are presented. In the pH range of 6 to 7 the MORD curve of HbA^{6pCMB} is identical with that of HbA: i.e. the value of the minimum at 586 nm is two times greater than that at 556 nm. At pH > 7 the value of the minimum at 586 nm begins to decrease showing that noncooperative subunits are produced. The midpoint of

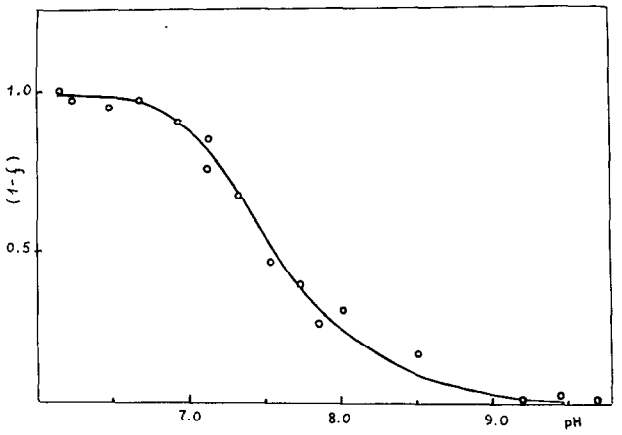

Fig. 2. Dissociation of HbA^{6pCMB}, at 20°; concentration of Hb 0.1%; in 0.2 M phosphate buffer + 0.1 M NaCl. $(1-f)$ is the fraction of undissociated molecules.

the curve $(1-f)$ versus pH is pH 7.5 (for Hb concentration 0.1%). According to sedimentation data [18] HbA^{6pCMB} is tetrameric in the pH range of 6 to 7 (at concentration ≈ 0.15–0.25%). At higher pH a sedimentation constant decreases from the value about 4.4 which is that for a tetramer, to a value of about 2.3, somewhat above that for a monomer, at pH 7.8. In the same pH range (midpoint is pH 7.3) the extinction coefficient of the Soret band changes from the value for native HbA to that for the isolated chains. So the initial species of HbA^{6pCMB} dissociation are tetramers and the final ones are either noncooperative dimers or isolated chains. If they are dimers it is likely that they are $\alpha_1\beta_2$ but not $\alpha_1\beta_1$ because both unreactive SH-groups Cys 104 α and Cys 112 β are involved in $\alpha_1\beta_1$ contacts.

The mechanism of HbA^{6pCMB} dissociation is now under study and the detailed data will be published later.

References

[1] M.E. Andersen, J.K. Moffat and Q.H. Gibson, J. Biol. Chem. 246 (1971) 2796.
[2] G.L. Kellett, J. Mol. Biol. 59 (1971) 401.
[3] N.A. Sharonova, Yu.A. Sharonov and M.V. Volkenstein, Biochim. Biophys. Acta 271 (1972) 65.
[4] G.L. Kellett, Nature New Biol. 234 (1971) 189.
[5] J.A. Hewitt, J.V. Kilmartin, L.P. Ten Eyck and M.F. Perutz, Proc. Natl. Acad. Sci. U.S. 69 (1972) 203.

[6] M.F. Perutz, J. Mol. Biol. 13 (1965) 646.
[7] A. Rossi-Fanelli, E. Antonini and A. Caputo, J. Biol. Chem 236 (1961) 391.
[8] M.A. Rosemeyer and E.R. Huehns, J. Mol. Biol. 25 (1967) 253.
[9] P.D. Boyer, J. Amer. Chem. Soc. 76 (1954) 4331.
[10] M.V. Volkenstein, Yu.A. Sharonov and A.C. Shemelin, Molek. Biol. USSR 2 (1968) 864.
[11] W. Bolton and M.F. Perutz, Nature 228 (1970) 551.
[12] Handbook of Biochemistry, 2nd ed., ed. H.A. Sober, USA.
[13] M.F. Perutz, M. Muirhead, J.M. Cox and L.C.G. Godmen, Nature 219 (1968) 131.
[14] E. Antonini and M. Bruneri, J. Biol. Chem. 244 (1969) 3909.
[15] E. Bucci and C. Fronticelli, J. Biol. Chem. 240 (1965) PC551.
[16] E. Chiancone, D.L. Currell, P. Vecchini, E. Antonini and J. Wyman, J. Biol. Chem. 245 (1970) 4105.
[17] Y.K. Yip and E. Bucci, J. Biol. Chem. 243 (1968) 5948.
[18] A. Antonini, E. Bucci, C. Fronticelli, E. Chiancone, J. Wyman and A. Rossi-Fanelli, J. Mol. Biol. 17 (1966) 29.