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THE pK OF THE AMINO TERMINAL GROUPS OF CARBONMONOXY- AND DEOXYHEMOGLOBIN MEASURED BY DINITROPHENYLATION IN PHOSPHATE BUFFERS

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The rate of reaction of the terminal valines of the α- and β-chains of hemoglobin with 1-fluoro-2,4-dinitrobenzene was followed spectrophotometrically at 353 nm. The variation with pH of the rate of dinitrophenylation of these groups was measured for both carbonmonoxy- and deoxyhemoglobin. In carbonmonoxyhemoglobin the results indicated a pK near 6.7 and 7.7 for the amino terminal groups of the two kinds of subunits, and were attributed to the α- and β-chains respectively. Removal of ligands produced an increase of 0.1 in both pK values and a decrease of 40% of the pH-independent kinetic constant for dinitrophenylation of the β-subunits. These modifications are due to the conformational changes associated with ligand binding in the system. In phosphate buffers the contribution to the Bohr effect of the amino terminal residues of either chains is negligible.

1. Introduction

Perutz [1] originally proposed that the Bohr effect of hemoglobin is in part produced by the α-amino groups of the α-chains (Val-α1) and by the terminal carboxyl residue of the β-subunits (His-β146).

Consistent with this proposition, Hill and Davis [2] have demonstrated a pK near 6.7 for the terminal valine of the α-subunits in liganded hemoglobin. In the laboratory of Gurd [5–7] this datum was confirmed and it was shown that this group acquires a pK near 7.8 upon removal of ligands. These experiments were performed in solvents which included chlorides, carbonates and cyanides, but not phosphates.

Recently, Russu and Ho [8] have shown that the pK of His-β146 shifts from 7.14 to 8.08 upon removal of ligands when hemoglobin is in phosphate buffer, as anticipated by Perutz [1]. However, they also showed that in Tris buffers the same group retains a pK near 7.9 in both liganded and unliganded hemoglobin.

In view of these findings, we thought it of interest to investigate the pK of the amino terminal groups of hemoglobin in the presence and absence of ligands in phosphate buffers. As shown for the isolated α-subunits of hemoglobin by De Bruin and Bucci [3], the substitution of the amino terminal valine with FDNB can be followed spectrophotometrically at 353 nm and is a very specific way of measuring its ionization. Lysines do not appear to be dinitrophenylated at neutral pH and histidines do not give stable derivatives with FDNB. Also, Neer and Konigsberg [4] have demonstrated that the SH groups of hemoglobin do not react with FDNB.

The data showed that removal of ligands produced only a negligible change of the pK values of the amino terminal groups of hemoglobin, suggest-

Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

ing that the α-amino groups of the α-chains are Bohr effect groups in chloride, cyanides and carbonates, as found by Gurd and his collaborators, but that they are not so in phosphate buffers.

2. Materials and methods

Adult human hemoglobin was prepared by the toluene procedure of Drabkin [9]. The stock solution of hemoglobin was deionized by recycling through a mixed bed resin column for 1 h. The concentration of hemoglobin solutions was measured using $\epsilon_{540} = 14\,000$ per heme for the carbonmonoxy derivative. FDNB was obtained from Sigma Chemical Co. and used without further purification. For all experiments freshly prepared FDNB solutions (≈ 10 mM) in 0.1 M NaCl were used. Their concentration was measured as described by Hill and Davis [2].

For both hemoglobin and carbonmonoxyhemoglobin the rate of reaction was measured by following the change in absorbance at 353 nm as described for the isolated α-chains [3]. 10 ml of a 2×10^{-4} M hemoglobin solution were mixed with 5 ml of 0.2 M phosphate buffer at the desired pH and 5 ml of 10^{-2} M FDNB in a jacketed vessel thermostatically maintained at 25°C. At regular time intervals, 2-ml samples were pipetted into test tubes containing 6 ml of 0.2 M phosphate buffer, pH 5.4, kept at 0°C in an ice bucket. In this way the reaction rate was lowered about 2000-fold and dialysis was started immediately at 4°C against three changes of 2 l of 0.02 M phosphate buffer at pH 5.4. Control FDNB solutions dialyzed in the same way showed a residual absorbance near 0.002 at 353 nm and were used as blanks in the spectrophotometric measurements. A Cary 14 spectrophotometer was used to measure the absorbance of the solutions.

For the reaction with hemoglobin, the FDNB solution was made oxygen free by bubbling nitrogen through and was mixed under anaerobic conditions with the deoxygenated hemoglobin solutions. Deoxyhemoglobin was converted to carbonmonoxyhemoglobin immediately after sample taking. The deoxygenation of the samples was checked spectrophotometrically.

All solutions were free of carbon dioxide.

Amino acid analyses were performed with a Beckman 120c autoanalyzer.

2.1. Treatment of the data

The reaction was followed spectrophotometrically, taking as described for the isolated α-chains [3],

$$1 - \frac{X}{P_0} = 1 - (R_0 - Rt)\epsilon_{540}/\epsilon_{353} \qquad (1)$$

where X is the amount of α-amino groups reacted, P_0 the total heme concentration, R_t the ratio of the absorbance at 540 to that at 353 nm of the sample at time t, R_0 the same ratio at time zero, $\epsilon_{540} = 14 \times 10^3$ the extinction coefficient per heme of carboxyhemoglobin at 540 nm, and $\epsilon_{353} = 16.2 \times 10^3$ the extinction coefficient of the valyl-dinitrophenyl derivative as evaluated by Neer and Konigsberg [4].

Since we are dealing with the substitution of the amino terminal residues of two different chains, the time dependence of $(1 - X/P_0)$ is described by the average of two exponentials:

$$1 - \frac{X}{P_0} = \tfrac{1}{2}\exp\left(-\frac{k_\alpha^0 K_\alpha}{[H^+] + K_\alpha}[F]t\right) + \tfrac{1}{2}\exp\left(-\frac{k_\beta^0 K_\beta}{[H^+] + K_\beta}[F]t\right) \qquad (2)$$

where k_α^0 is the time constant of the reaction for the amino terminal groups of the α-chains and k_β^0 that of the β-chains. In the same way, K_α and K_β refer to the ionization constant of the amino terminal groups of the α- and β-chains, respectively, [F] is the concentration of FDNB and t the time in seconds.

As shown in fig. 1, a plot of $\log(1 - X/P_0)$ vs. t was essentially linear at all pH values considered up to at least 70–80% completion of the reaction. This indicated that the two exponentials in eq. 2 were sufficiently similar so as to make it degenerate into a single exponential of the kind

$$\log\left(1 - \frac{X}{P_0}\right) = -\frac{1}{2}\left(\frac{k_\alpha^0 K_\alpha}{[H^+] + K_\alpha} + \frac{k_\beta^0 K_\beta}{[H^+] + K_\beta}\right)[F]t = -K_H^{OD}[F]t \qquad (3)$$

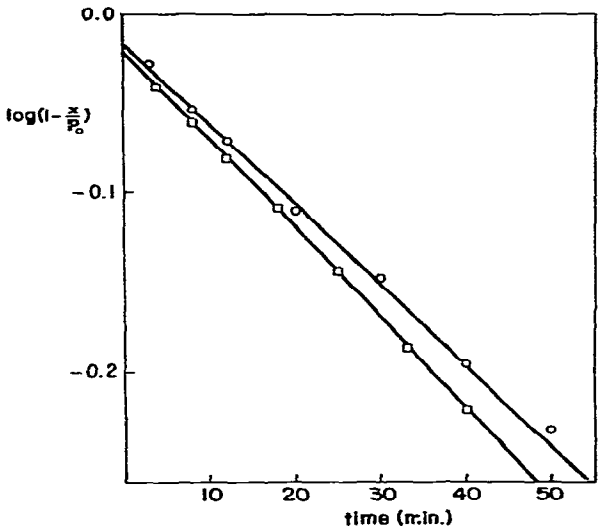

Fig. 1. Absorbance measurements of the reaction of hemoglobin with a 25-fold excess of FDNB in 0.05 M phosphate buffer at 25°C. (○) First-order plot for the reaction with carbonmonoxyhemoglobin at pH 6.640. (□) The same plot for the reaction with hemoglobin at pH 7.005.

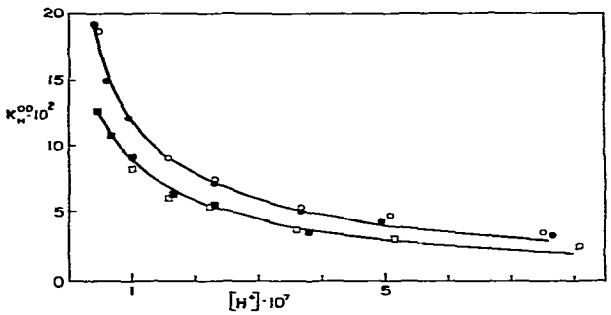

Fig. 2. pH dependence of the reaction of hemoglobin with FDNB, measured spectrophotometrically. Upper curve shows the results for carbonmonoxyhemoglobin, lower curve those for hemoglobin. Different symbols refer to different experiments. The continuous lines were calculated with the parameters listed in table 1.

Simulation experiments in which the parameters listed in table 1 were used confirmed that in the whole pH range investigated, the plot of $\log(1 - X/P_0)$ vs. t was linear and that the slope of the line corresponded to the average of the exponents in eq. 3 to within 1–2%. It was also shown that in cases where the ratio between the two exponents was choosen to be 10 the initial slopes (up to 50% of the reaction) corresponded to the average of the exponents to within 3%.

For these reasons, K_H^{obs} was calculated from the slope of the plot of $\log(1 - X/P_0)$ vs. t at several pH values and the parameters involved in eq. 3 were estimated with nonlinear least-square procedures based either on the recursive method of Kalman (as reported by Walker and Duncan [10]), or on the maximum neighborhood method of Marquardt [11]. The two procedures gave essentially identical results.

3. Results and discussion

The parameters listed in table 1 for deoxyhemoglobin show that deoxygenation produced an approx. 40% decrease in k_β^0 and a 25% decrease in the two K values. This makes a difference of only 0.1 in the corresponding pK values.

It is worth considering the possibility that other groups besides the amino termini of the α- and β-chains of hemoglobin participated in the reaction with FDNB and what effect this might have produced in our analyses. Extensive investigation of the products of the reaction of hemoglobin and

Table 1

Kinetic parameter estimated with the recursive procedure of Kalman (see ref. 10) for carbonmonoxy- and deoxyhemoglobin

Derivative	k_α^0	$K_\alpha(\times 10^7)$	pK_α	k_β^0	$K_\beta(\times 10^7)$	pK_β
Carbonmonoxyhemoglobin	0.20	2.042	6.69	0.64	0.199	7.70
Hemoglobin	0.19	1.585	6.80	0.38	0.158	7.80

of the isolated α-chains with FDNB at neutral pH has been performed by Neer and Konigsberg [4] and De Bruin and Bucci [3]. Concerning the histidines, they showed that no stable dinitrophenyl derivatives were formed. Also, the cysteines at β93 did not appear to react with FDNB at neutral pH [2]. In any case, our spectrophotometric measurements would have been negligibly affected by the formation of dinitrophenyl-substituted histidines or cysteines because of their low absorption. With respect to the lysines, the results of Neer and Konigsberg [4] and De Bruin and Bucci [3] indicate only a negligible participation of these residues in the reaction under the chosen conditions. A few experiments of chromatographic fractionation of the reacted hemoglobin followed by amino acid analyses confirmed that no meaningfull reaction of lysine residues occurred in the cases presented here. Also, the experiments were carried out in a pH range far from the pK of lysine residues, so that their participation to the reaction would have been practically independent of pH and in any case identical for both carbonmonoxy- and deoxyhemoglobin.

It has to be stressed that a possible conformational change of hemoglobin due to dinitrophenyl substitution of the terminal amino group of the chains does not interfere with the results reported because the K_H^{OD} values were obtained in the early stage of the reaction, when the chance that a molecule is substituted at more than one terminal is very small, and a conformational change triggered by the substitution of only one group will probably not affect the pK determination of that group. Also, this procedure was capable of detecting pK values near 7.3 [3], proving its applicability to the problem here investigated.

The gross phenomenon appearing in our data is the small difference in the overall rates of reaction of liganded and unliganded hemoglobin at all of the pH values investigated. It should be stressed that a 10-fold difference in the K values was necessary for producing a pK shift of 1 unit.

There is no room for such a change in our data. This was also investigated making simulations by hand rather than by computer. If the pK of one group was increased by more than 0.2 units, the pK of the other group had to be lowered in order to obtain acceptable simulations of the data.

A pK shift of 0.1 upon removal of ligands may result from the different isoionic points of liganded and unliganded hemoglobin.

With regard to the identification of the groups, a pK of 6.7 assigned to the α1 valine is very consistent with the pK estimated by Hill and Davis [2]. The pH-independent kinetic constant found in our simulations is also very consistent with the value reported by these authors. Also, Garner et al. [5] found a value near 6.8 for the α1 valine in carbonmonoxyhemoglobin in the presence of chlorides.

In so doing, we are assigning a pK near 7.7 to the β1 valine. This value is reasonable for an amino terminal residue and is higher than the value (near 6.9) determined in the laboratory of Gurd [5–7] for this group using either kinetics or NMR techniques. In those experiments, the protein was in the presence of chlorides and carbonates. In our experiments, carbonates had been carefully eliminated by using deionized proteins and deionized water for preparing the various solutions. Also, phosphates and not chlorides were used. The discrepancy may again reflect the influence of different solvents on the proton binding behaviour of charged groups in hemoglobin.

It appears that the α1 valine is a Bohr effect group in chloride and carbonate ions [5–7], but not in phosphates. The reverse is true for the β146 histidine, which is a Bohr effect group in phosphates but not in chlorides [8]. Thus, different groups produce the Bohr effect in different solvents. The question may be posed as to whether or not the Bohr effect is a phenomenon linked to certain specific groups on the hemoglobin surface.

We defer this discussion to a paper now in preparation.

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