Chemical properties of the *N*-termini of human haemoglobin

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The chemical properties, namely *pK* and reactivity, of the *N*-termini of oxyhaemoglobin and deoxyhaemoglobin toward acetic anhydride and 1-fluoro-2,4-dinitrobenzene (Dnp-F) were determined by the competitive-labelling approach [Kaplan, Stevenson & Hartley, (1971) *Biochem. J.* **124**, 289–299; Duggleby & Kaplan (1975) *Biochemistry* **14**, 5168–5175]. At physiological pH and temperature, the valine-1 α and valine-1 β amino groups had unusually low *pK* values, but showed only minimal changes in their *pK* values on deoxygenation. Between pH 7.5 and pH 8.0 a deviation was observed in the pH–reactivity profiles and the apparent *pK* values became markedly pH-dependent. It was found that Dnp-F, but not acetic anhydride, had an abnormally high reactivity toward the *N*-termini. It is concluded that the valine-1 α and valine-1 β *N*-termini make little or no contribution to the alkaline Bohr effect at physiological pH values. The high reactivity toward Dnp-F is attributed to an interaction or binding near the *N*-terminal region, and the discontinuity in the pH–reactivity profile at moderate alkaline pH values to a conformational change which alters the environment of these groups.

On the basis of X-ray-crystallographic studies (Perutz, 1970), the structural basis for the alkaline Bohr effect in haemoglobin has been attributed in part to increases in ionization constants of the valine *N*-terminus of the α -chain and the imidazole group of histidine-146 of the β -chain. The X-ray structure was interpreted as showing that these residues are involved in salt bridges in deoxyhaemoglobin but not oxyhaemoglobin. It was inferred from this evidence that these groups in deoxyhaemoglobin would have substantially higher *pK* values and therefore contribute to the increased basicity. In a series of studies using carbon-13 n.m.r. spectroscopy (Morrow *et al.*, 1974), kinetic (Garner *et al.*, 1975) and theoretical (Matthew *et al.*, 1977) approaches, Gurd and his co-workers reported that the ionization constant of the valine-1 α *N*-terminus in deoxyhaemoglobin is increased by approximately one *pK* unit over that in the liganded form of haemoglobin. Other studies, by Ohe & Kajita (1980), who used ^2H exchange, supported the prediction that histidine-146 β has a substantially higher *pK* in non-liganded haemoglobin. However, it was found by those authors that the entire Bohr effect at physiological pH value could be accounted for by the shift in ionization

constant of histidine-146 β and histidine-89 α . Inclusion of the reported contribution of valine-1 α gave an overall H^+ uptake greater than that actually observed on deoxygenation of haemoglobin. On the basis of this evidence, it appears that the underlying molecular basis for the Bohr effect may not be as clear-cut as proposed by Perutz (1970).

Unusual chemical properties have been reported from chemical-reactivity studies on the *N*-termini of the α - and β -chains of haemoglobin. Garner *et al.* (1975), using a cyanate probe, found that in liganded haemoglobin valine-1 α and valine-1 β had *pK* values such that at physiological pH these groups would be deprotonated. Furthermore, valine-1 α was found to have an abnormally high reactivity in the non-liganded state but normal reactivity in the liganded state. In an earlier study, Hill & Davis (1967), using Dnp-F as reagent, also reported an unusually low *pK* value for valine-1 α in this liganded state but, unlike Garner *et al.* (1975), found this group to be abnormally reactive. The present investigation was undertaken with a view to resolving some of the inconsistencies in the literature by carrying out a more detailed and rigorous kinetic approach to determining the *pK* values of putative Bohr groups. Reactivity studies were carried out at 37°C over a wider range of pH than previously reported, with two different chemical probes, acetic anhydride and

Abbreviations used: Dnp-F, 1-fluoro-2,4-dinitrobenzene; Dnp-Val, dinitrophenylvaline.

Dnp-F, by means of the competitive-labelling approach of Kaplan *et al.* (1971).

Experimental

Materials

DEAE-Sephadex A-50 was obtained from Pharmacia (Canada) Ltd. (Dorval, Que., Canada). All fine chemicals were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.), whereas the radioactive reagents came from Amersham Corp. (Oakville, Ont., Canada). Aquasol-2 was obtained from NEN Canada (Lachine, Que., Canada); the silica-gel thin-layer plates were from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Haemoglobin preparation

A modified Drabkin's (1946) procedure was used. Freshly drawn blood from one of us (P. A. H.) was centrifuged at 9000 *g* for 15 min at 4°C to pack the red cells. These cells were washed twice with 0.9% NaCl at 4°C. Lysis of the cells was achieved by vigorous shaking after addition of 2 vol. of water and $\frac{1}{2}$ vol. of toluene. After leaving the solution for 1 h, it was centrifuged at 10000 *g* for 30 min at 4°C. The cellular membranes were separated from the haemoglobin by filtration through Whatman no. 1 filter paper.

To obtain phosphate-free haemoglobin A, the method of Williams & Tsay (1973) was used. The stock haemoglobin solutions, one containing equimolar haemoglobin and phenylalanine for acetylation, the other, equimolar haemoglobin and L- β -imidazole/lactic acid for dinitrophenylation, were adjusted to a concentration of 38.7 mg of haemoglobin/ml (determined from A_{540}) in a buffer containing 5 mM-*N*-methylmorpholine, 5 mM-boric acid and 0.1 M-KCl.

Trace isotopic labelling

For each labelling experiment, haemoglobin stock solution was diluted to a concentration of 1 mg/ml and 3.5 ml of this was used. To prepare oxy-haemoglobin, pure O₂ was bubbled through the solution before labelling and an O₂ atmosphere maintained during the labelling. Deoxygenation was achieved by bubbling N₂ through the solution for 10 min and then maintaining an N₂ atmosphere above the reaction mixture during labelling. Complete deoxygenation was verified by observing the shifts in A_{545} and A_{577} .

(1) [³H]Acetic anhydride. Each portion of the haemoglobin and phenylalanine stock solution was adjusted to the desired pH with 1 M-KOH and maintained at 37°C in a thermostatically controlled water jacket. A portion of [³H]acetic anhydride in 25 μ l of acetonitrile (189 nmol; sp. radioactivity 6.6 Ci/mmol) was added to the reaction mixture with

rapid stirring. The reaction was terminated after 5 min by adjusting the pH to 2 with HCl. After addition of 3.5 g of urea to denature the reaction mixture, full acetylation was achieved by reaction with unlabelled acetic anhydride (50 μ l), a pH-stat being used to maintain the pH at 8.0.

(2) [³H]Dnp-F. Each sample of haemoglobin and L- β -imidazole solution was adjusted to the desired pH with 1 M-KOH and maintained at 37°C in a water bath. A portion of [³H]Dnp-F in 25 μ l of acetonitrile (1.72 nmol; sp. radioactivity 14.5 Ci/nmol) was added with rapid stirring and then incubated at 37°C for 16 h, after which time the reaction was terminated by adjusting the pH to 2 with HCl. Full dinitrophenylation was achieved by addition of 0.5 g of NaHCO₃ and 50 μ l of unlabelled Dnp-F [in acetonitrile (50%, v/v)] and shaking at 22°C for 16 h.

¹⁴C-labelling

(1) [¹⁴C]Acetic anhydride. A 4 ml portion of haemoglobin stock solution containing phenylalanine was made 8 M with respect to urea. To this was added 2.5 mmol of [¹⁴C]acetic anhydride (sp. radioactivity 100 μ Ci/mmol) with rapid stirring. The pH was maintained at 9 with a pH-stat, 5 M-KOH being used as titrant. The acetylated reaction mixture was diluted to 10 ml and 0.50 ml was added to each ³H-labelled sample. The pH was adjusted to 2 with conc. HCl.

(2) [¹⁴C]Dnp-F. Haemoglobin stock solution containing L- β -imidazole/lactic acid was diluted so that there was 60 mg of haemoglobin in a 30 ml solution of 10% (w/v) NaHCO₃ and 8 M-urea. To this was added 550 μ mol of [¹⁴C]Dnp-F (sp. radioactivity 200 μ Ci/mmol) and reaction allowed to proceed for 16 h at 22°C with shaking. This reaction mixture was diluted to 50 ml and 2.0 ml was added to each ³H-labelled sample. The pH was adjusted to 2 with conc. HCl.

Purification of ³H, ¹⁴C-labelled derivatives

(1) [³H, ¹⁴C]Acetic anhydride. The acetylphenylalanine was extracted from the derivatized haemoglobin with 3 $\times$ 5 ml vol. of ethyl acetate. After drying, purification of the internal standard was carried out as described previously (Kaplan, 1972).

The [³H, ¹⁴C]acetylated haemoglobin in the aqueous phase was dialysed against water, to remove urea, and freeze-dried. Fig. 1 illustrates the enzymic digestions used to isolate the desired peptides by procedures described previously (Sheffer & Kaplan, 1979; Visentin & Kaplan, 1975).

(2) [³H, ¹⁴C]Dnp-F. The ³H, ¹⁴C-labelled samples were extracted with several volumes of diethyl ether to remove excess dinitrophenol and to aid precipitation of protein. Centrifugation at 1000 *g* for 10 min separated the supernatant, which contained

Fig. 1. Enzymic digestions and composition of the isolated peptides of acetylated haemoglobin. The sequences are taken from established sequences (Dayhoff, 1972). Abbreviation used: Ac, acetyl.

the imidazole-[³H,¹⁴C]Dnp-lactic acid from the [³H,¹⁴C]protein. The precipitate from the aqueous phase was washed twice with 0.5 ml portions of water. After adding these washings to the supernatant phase, the internal standard was purified by using the method of Chan *et al.* (1981).

In order specifically to isolate [³H,¹⁴C]Dnp-Val from the α-chain, advantage was taken of the observation of Rhinesmith *et al.* (1957) that Dnp-Val-Leu can be quantitatively isolated by a brief acid hydrolysis. The dried [³H,¹⁴C]Dnp-haemoglobin was hydrolysed with 6 M-HCl in an oil bath at 110°C for 30 min. The hydrolysate was extracted with ether. The remaining hydrolysate was rehydrolysed for 1 h and then further extracted with ether. After combining these ether extracts, the Dnp-Val and Dnp-Val-Leu were separated by silica-gel t.l.c. by using an overflow technique with benzene/pyridine/acetic acid (40:10:1, by vol.) (Dnp-Val-Leu/Dnp-Val = 1.56). The Dnp-Val-Leu was eluted with acetone/water (8:1, v/v), dried and then hydrolysed in 6 M-HCl *in vacuo* for 16 h at 110°C. The Dnp-Val, which must be derived from the α-chain, was rechromatographed on silica gel, eluted and dried.

After removing traces of ether from the 1½ h hydrolysate, a further hydrolysis was performed *in vacuo* at 110°C for 24 h. This hydrolysate was extracted with ether and purified by t.l.c. as described above. No Dnp-Val-Leu was present, and the [³H,¹⁴C]Dnp-Val, which is derived from the β-chain, was eluted and dried.

pH measurements and titrations

A Radiometer 26 pH-meter fitted with a type GK

2321C glass electrode was used for pH measurements. Titrations were performed by adding titrant from an Alga micrometer-syringe apparatus.

Radioactivity measurements and calculations

All dried samples were dissolved in 50 μl of water. After addition of 10 ml of Aquasol-2, they were counted on a LKB RackBeta scintillation counter equipped for automatic quench correction and d.p.m. conversion. The second-order velocity constants for the reaction of each functional group relative to the internal standard were calculated by using the following equation (Kaplan *et al.*, 1971):

$$\alpha_x r = \alpha_s ({}^3\text{H}_x/{}^{14}\text{C}_x) / ({}^3\text{H}_s/{}^{14}\text{C}_s) \quad (1)$$

where α_x is the degree of ionization of the functional group being studied, α_s is the degree of ionization of the internal standards [imidazole lactate $\text{p}K = 7.27$ (Cruickshank & Kaplan, 1975); phenylalanine $\text{p}K = 9.01$ (Sheffer & Kaplan, 1979)], r is the second-order pH-independent velocity constant for the reaction of the functional group under study relative to that for its internal standard and ${}^3\text{H}_x/{}^{14}\text{C}_x$ and ${}^3\text{H}_s/{}^{14}\text{C}_s$ are the corresponding ³H/¹⁴C ratios for the group and internal standard respectively.

Results

Competitive labelling with acetic anhydride

Fig. 1 summarizes the data from the isolation of peptides derived from fully acetylated haemoglobin, which was used in making assignments of reactivities to individual functional groups. A pepsin digest yielded peptides 1α-11α and 1β-8β. As these peptides contained three and two amino groups

respectively, further fragmentation was required in order to make an unequivocal assignment of parameters to individual functional groups. In the case of the α -chain peptide, digestion with Pronase yielded two peptides, 1 α -5 α and 6 α -7 α , each containing a single derivatized group and N^ϵ -acetyllysine, which could be derived from either the 7 α - or 11 α -positions. The β -chain peptide yielded two peptides, 1 β -2 β and 4 β -8 β , each with a single derivatized group.

Fig. 2. pH-reactivity profile at 37°C for the reaction with acetic anhydride of valine-1 α in (a) deoxyhaemoglobin and (b) oxyhaemoglobin

The continuous lines are theoretical curves with the parameters given in Table I obtained by using the relationship:

$$\alpha_x r = \frac{r}{1 + \frac{[H^+]}{K_a}}$$

The reactivity data obtained for the N -terminus of the α -chain is plotted in Fig. 2(a) for deoxyhaemoglobin and Fig. 2(b) for oxyhaemoglobin. Analysis of these data showed that it did not fit a theoretical titration curve over the entire range of pH studied. Theoretical titration curves (continuous lines) could be fitted to the data in the lower pH range. Linearization of the data (Fig. 3) shows more clearly that only the data at the lower pH values fit titration curves and that there is a deviation in the pH profiles just below pH 8, where the reactivity increases in each case. Deoxygenation does not markedly affect the ionization constant of this group in the lower pH region studied. However, it may be significant that the deviation in the deoxygenated form occurs near pH 7.25, and near pH 7.5 in the oxygenated form. At the pH values above the deviation, the apparent pK and the reactivity are increasing and are pH-dependent.

The results for the N -terminus of the β -chain (Fig. 4) are very similar to those of the α -chain. In both the deoxygenated and the oxygenated forms there is a similar deviation, and also like the α - N -terminus there is a slight upward shift in pK and an increase in reactivity of the deoxygenated form. Fig. 5 shows where these groups lie in relation to a Bronsted plot

Fig. 3. Linear plot, from the data from Fig. 2, for the reaction of acetic anhydride with valine-1 α in (a) deoxyhaemoglobin and (b) oxyhaemoglobin

Fig. 4. pH-reactivity profile at 37°C for the reaction with acetic anhydride of valine-1 β in (a) deoxyhaemoglobin and (b) oxyhaemoglobin

for the reaction of primary amines with acetic anhydride (Kaplan *et al.*, 1971).

Since the lysine ϵ -amino groups in the terminal regions of the α - and β -chains had pK values greater than 9 (Fig. 6), it was only possible to estimate the pK values and absolute reactivity by extrapolation of the data. The pH-reactivity profiles for these groups show a slightly decreased reactivity in the deoxygenated state but no discontinuous or abnormal properties in terms of the Bronsted relationship (Fig. 5).

Competitive labelling with Dnp-F

The pH-reactivity profiles obtained for the α - and β -N-termini are shown in Figs. 7 and 8. As with

Fig. 5. Positions of the amino groups in the N-terminal region of deoxyhaemoglobin and oxyhaemoglobin on a Bronsted plot for the reaction of primary amines with acetic anhydride

Abbreviations used: Hb, deoxyhaemoglobin; HbO₂, oxyhaemoglobin; Bz, benzoyl.

acetic anhydride, there are deviations at about pH 8 in all the profiles, but rather than the reactivity increasing, it decreases after the deviation. Again there is no marked shift in the ionization constant of either group on deoxygenation, but the absolute reactivity of the deprotonated group decreases rather than increasing. The pK values obtained in both cases are in good agreement and are summarized in Table 1. Inclusion of 2,3-diphosphoglycerate at 5×10^{-3} M in the reaction mixture, at physiological pH values, had no significant effect on the reactivity of these groups (Figs. 7 and 8). Fig. 9 shows where these groups are located on a Bronsted plot for the reaction of primary amines with Dnp-F (Kaplan *et al.*, 1980). It is clear that the reactivities of these groups are approximately an order of magnitude greater than expected.

Discussion

Two previous kinetic studies have been carried out with the objective of directly determining the ionization constants of the N-termini of liganded and deoxyhaemoglobin, thereby quantifying their contribution to the alkaline Bohr effect. The first, by Hill & Davis (1967), used Dnp-F as the chemical probe, and the other, by Garner *et al.* (1975), used cyanic acid. Their results, along with those obtained in the

Fig. 6. *pH-reactivity profiles at 37°C for reaction with acetic anhydride of the ϵ -amino group of lysine-7 α (....) and lysine-8 β (—) in (a) deoxyhaemoglobin and (b) oxyhaemoglobin*

present study and other indirect estimations of pK values, are summarized in Table 1. There are many areas of general agreement between our results and those of others, but the notable exception is that we do not observe any shift in the ionization constant of the valine-1 α amino group on deoxygenation with either acetic anhydride or Dnp-F as the chemical probe.

Fig. 7. *pH-reactivity profile at 37°C for the reaction with Dnp-F of valine-1 α in (a) deoxyhaemoglobin and (b) oxyhaemoglobin*

●, Haemoglobin in 5 mM-2,3-diphosphoglycerate.

Hill & Davis (1967) reported an unusually low pK value and an abnormally high reactivity for the valine-1 α *N*-terminus of carbonmonoxyhaemoglobin toward Dnp-F at 25°C in 0.1 M-KCl. As shown in the Bronsted plot (Fig. 9), our results with oxyhaemoglobin at 37°C confirm these conclusions. The deviation we observed at approx. pH 8 (Figs. 7 and 8) was not noted by Hill & Davis (1967), since they did not report any data above this pH value. Although they mention that they had evidence that the pK of the valine-1 α *N*-terminus is increased on deoxygenation, this evidence has not appeared in published form. Those authors could not

Table 1. Chemical properties of amino groups of haemoglobin

The values of p*K* and reactivity, and their standard errors, were calculated by fitting the data to theoretical titration curves by using a non-linear least-squares procedure. In the column headed 'Reactivity', *r* is the ratio of the second-order rate constants for the group under study to its internal standard; *k* is the second-order rate constant (M⁻¹·s⁻¹). Abbreviation used: Ac₂O, acetic anhydride.

Functional group	Reagent	Deoxyhaemoglobin		Liganded haemoglobin (O ₂ or CO)	
		p <i>K</i>	Reactivity	p <i>K</i>	Reactivity
Valine-1α	Ac ₂ O	7.11 ± 0.09	<i>r</i> = 0.0756 ± 0.0089	6.92 ± 0.05	<i>r</i> = 0.0519 ± 0.0021
	Dnp-F	7.09 ± 0.05	<i>r</i> = 184 ± 6	7.03 ± 0.08	<i>r</i> = 205 ± 12
	Cyanic acid*	7.79 ± 0.10	<i>k</i> = 91 ± 14	6.95 ± 0.13	<i>k</i> = 12 ± 1.5
	Dnp-F†	—	—	6.72 ± 0.03	<i>k</i> = 0.236 ± 0.009
	CO ₂ ‡	7.83 ± 0.19	—	7.16 ± 0.36	—
Valine-1β	CO ₂ §	7.05 ± 0.31	—	7.16 ± 0.17	—
	Ac ₂ O	6.90 ± 0.06	<i>r</i> = 0.0814 ± 0.0035	6.77 ± 0.06	<i>r</i> = 0.0408 ± 0.0018
	Dnp-F	6.93 ± 0.03	<i>r</i> = 54.9 ± 1.0	7.14 ± 0.06	<i>r</i> = 75.9 ± 3.3
	Cyanic acid*	6.84 ± 0.12	<i>k</i> = 24 ± 2	7.05 ± 0.05	<i>k</i> = 18 ± 1.6
	CO ₂ ‡	6.91 ± 0.32	—	—	—
Lysine-7α	CO ₂ §	6.13 ± 0.44	—	7.16 ± 0.17	—
	Ac ₂ O	9.83 ± 0.13	<i>r</i> = 0.774 ± 0.200	10.25 ± 0.10	<i>r</i> = 1.87 ± 0.40
Lysine-8β	Ac ₂ O	9.46 ± 0.21	<i>r</i> = 0.373 ± 0.140	9.61 ± 0.07	<i>r</i> = 0.599 ± 0.079

* Garner *et al.* (1975).
† Hill & Davis (1967).
‡ Matthew *et al.* (1977).
§ Gros *et al.* (1981).

Fig. 8. pH-reactivity profile at 37°C for the reaction with Dnp-F of valine-1β in (a) deoxyhaemoglobin and (b) oxyhaemoglobin
●, Haemoglobin in 5 mM-2,3-diphosphoglycerate.

Fig. 9. Positions of the amino groups of deoxyhaemoglobin and haemoglobin on a Bronsted plot for the reaction of primary amines with Dnp-F
Abbreviations used: Hb, deoxyhaemoglobin; HbO₂, oxyhaemoglobin; Bz, benzoyl.

account for the abnormally high reactivity that we also observed. An explanation of this phenomenon becomes evident after an examination of the data we obtained with acetic anhydride.

The results with acetic anhydride also show no significant shift in pK value of the valine-1 α and valine-1 β N -termini on deoxygenation. As with Dnp-F, there is a change in the absolute reactivity of the deprotonated nucleophile on deoxygenation, indicating that some conformational change has taken place which affects the accessibility of these groups to the reagent. However, the change appears to be very limited, as the lysine ϵ -amino groups near the N -termini show little change in chemical properties (Table 1). The notable difference is that above the discontinuity in the pH-reactivity profiles the reactivities toward acetic anhydride and Dnp-F proceed in opposite directions. Examination of the Bronsted plots (Figs. 5 and 9) provides an explanation for this observation as well as for the high reactivity towards Dnp-F. The data obtained with acetic anhydride lie on a Bronsted plot, indicating that these groups behave as free amino groups in solution. If the high reactivity towards Dnp-F results from enhanced nucleophilicity conferred on the amino groups by the protein structure, then it would be expected that these groups would be abnormally reactive towards all reagents. The fact that they are not means that the high reactivity toward Dnp-F results from its interaction or binding with the protein, i.e. a site-directed reaction. The most probable explanation for the discontinuity in the reactivity profiles is a conformational change. This would result in a disruption of the environmental effects, e.g. proximity of positive charges, which confer the unusually low pK values on the groups. It is evident from the linear plots of the reactivity data for acetic anhydride (Fig. 3) that above pH 8 the pK and reactivity are increasing, as would be expected from such a conformational change. In the case of Dnp-F, this increased reactivity is more than compensated for by the loss of the site direction for Dnp-F, so that a net decrease is observed.

In the study by Garner *et al.* (1975), who used cyanate as the chemical probe, there is general agreement with our results regarding the low pK values for the N -termini, except for the valine-1 α of deoxyhaemoglobin. Like Hill & Davis (1967), they also did not note any deviation in the reactivity data. Although they state that data were obtained up to pH 8.7, none was reported above pH 7.68. As noted above, the deviation in the data occurs at pH 7.25 in deoxyhaemoglobin (Fig. 2). If one ignores the deviation, it is evident from Fig. 3 that the pK value obtained will be very dependent on the pH range selected for pK calculation. By using the data reported by Garner *et al.* (1975) between pH 6.6 and 7.1, we calculate a pK of 7.0 for valine-1 α in deoxyhaemoglobin, a result that is in excellent agreement with that obtained in the present study. If we included our data obtained above pH 7.25, we would obtain a pK value which would agree closely

with the higher value reported by Garner *et al.* (1975). It is our contention that the data above the pH where the deviation occurs should not be included, since a conformational change has taken place, altering the native structure. Garner *et al.* (1975) also report an abnormally high reactivity of the valine-1 α amino group of deoxyhaemoglobin toward cyanate. It is not clear why this should occur only in this case; however, because a charged reagent was employed, electrostatic interactions with the protein may occur, making any clear-cut interpretation difficult. Another possible explanation is that, unlike the trace-labelling conditions employed in the present study, the reaction with cyanate was carried out under conditions in which there was also substantial reaction of the valine-1 β N -terminus. As reaction of this group with CO_2 is known to alter the oxygen-binding properties of the haemoglobin molecules, it is possible that reaction of the valine-1 β N -terminus with cyanate alters the chemical properties, viz. pK and reactivity, of the valine-1 α N -terminus.

At present most of the evidence supporting the assignment of valine-1 α as a Bohr group is indirect in nature. The X-ray evidence (Perutz, 1970) provides valuable insights, but by itself is inconclusive, since small changes in the orientation of groups in solution can result in large changes in chemical properties. As pointed out by Kilmartin & Rossi-Bernardi (1973), the evidence that chemical modification of the N -terminal groups decreases the Bohr effect suffers from the fact that changes in other portions of the molecule cannot be ruled out. The more direct approaches involving chemical modification used by Garner *et al.* (1975), Matthew *et al.* (1977) and Gros *et al.* (1981) suffer from the same uncertainty. In fact in the two reported studies of the reaction of CO_2 with the N -termini, different conclusions are reached regarding the role of these groups in the Bohr effect. Matthew *et al.* (1977) conclude that valine-1 α is a Bohr group, whereas the pK values reported by Gros *et al.* (1981) lead one to the conclusion that valine-1 β and not valine-1 α is a Bohr group. Another competitive-labelling study by Bresciani (1977), who used reductive methylation, eliminates the uncertainty caused by chemical modification, but the decrease in reactivity of the valine-1 α N -terminus on deoxygenation that they reported at pH 7.01 could simply be due to a decrease in reactivity alone and not due to an upward shift in pK . The assumption that the valine-1 α has the same reactivity in oxyhaemoglobin and deoxyhaemoglobin is not justified.

The conclusion of the present study is that, at physiological pH values, neither the valine-1 α nor valine-1 β amino groups make a significant contribution to the Bohr effect. This result is in agreement with the observation of Ohe & Kajita (1980) that the

entire Bohr effect at physiological pH values can be accounted for by the ionization of two histidine residues. Our data do not rule out the possibility that above pH 7.5 the valine-1 α groups make a significant contribution. In order to verify this, extremely accurate data would be required, since the pK values of the amino groups are strongly pH-dependent above this pH. It could, in fact, be argued that the pK values of these groups are pH-dependent over the entire range of pH values employed in the present study. However, it appears from our data that the pH-dependence below pH 7.5 is minimal, so that a reasonably accurate estimation of pK can be made at physiological pH values.

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