Biochemistry

 Volume 26, Number 20 October 6, 1987

Perspectives in Biochemistry

How Much Do We Know about the Bohr Effect of Hemoglobin?†

Chien Ho* and Irina M. Russu‡

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, and Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06457

Received June 10, 1987; Revised Manuscript Received July 6, 1987

This paper summarizes the current state of our knowledge of the relationship between the Bohr effect and the hemoglobin (Hb)[1] structure. It does not aim to be comprehensive; rather, it is focused on recent experimental results and various interpretations of this subject. For detailed descriptions of the Bohr effect, refer to the following reviews: Wyman (1964); Perutz (1970); Antonini and Brunori (1971); Edsall (1972); Kilmartin and Rossi-Bernardi (1973).

The oxygen affinity of Hb, at pH above 6, increases with pH (the alkaline Bohr effect). Conversely, oxygenation results in the release of hydrogen ions by the Hb molecule. At pH below 6, the O_2 affinity increases with decreasing pH and the Hb molecule absorbs H^+ ions upon oxygenation (the acid Bohr effect).

The Bohr effect can be measured by two independent methods. First, it can be measured from the difference between the pH titration curves of oxyhemoglobin (HbO_2) or (carbonmonoxy)hemoglobin (HbCO)[2] and deoxyhemoglobin (deoxy-Hb). Second, it can also be measured from the change in O_2 affinity as a function of pH. According to the linkage theory of Wyman (1948, 1964), there is an exact relationship between the change in O_2 affinity and the number of protons released as a function of pH. The maximum value for the number of protons released per O_2 bound is approximately 0.5 at pH ~7.4. Anions [such as chloride, phosphate, 2,3-diphosphoglycerate (2,3-DPG), and inositol hexaphosphate (IHP)] exert a profound influence on the Bohr effect (Antonini et al., 1962, 1963, 1965; Kilmartin & Rossi-Bernardi, 1969; de Bruin et al., 1973, 1974a,b; Rollema et al., 1975; van Beek et al., 1978, 1979; van Beek & de Bruin, 1980; Ho & Russu, 1978; Russu et al., 1980, 1982; Benesch et al., 1969, 1986; Bonaventura & Bonaventura, 1978; Fronticelli et al., 1984; Bucci & Fronticelli, 1985).

There are two schools of thought regarding the molecular basis of the Bohr effect. One believes that there are only a limited number of amino acid residues (such as three to four per $\alpha\beta$ dimer) in the Hb molecule responsible for the alkaline Bohr effect [for example, see Perutz et al. (1980) and references cited therein]. The other believes that a large number of amino acid residues in Hb change their pK values in going from the deoxy to the oxy form and thus are potential Bohr groups [for example, see Russu et al. (1982) and references cited therein]. Moreover, any amino acid residues that have a differential affinity for anions between deoxy-Hb and HbO_2 could also contribute to the Bohr effect [for example, see van Beek et al. (1979) and references cited therein]. The implication of this suggestion is that different sets of amino acid residues are involved in the Bohr effect under different experimental conditions.

There are two main aims in this review. First, we shall summarize and evaluate four basic techniques that have been used to identify which amino acid residues are the Bohr groups. Second, we shall analyze the effects of anions on the Bohr effect.

Mutant and Chemically Modified Hbs

On the basis of a comparison of the atomic models of deoxy-Hb A and oxy-like horse methemoglobin (met-Hb), Perutz

† The hemoglobin research is supported by a research grant from the National Institutes of Health (HL-24525 to C.H.).

* Address correspondence to this author at Carnegie Mellon University.

‡ Wesleyan University.

[1] Abbreviations: Hb, hemoglobin; deoxy-Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, (carbonmonoxy)hemoglobin; met-Hb, methemoglobin; Hb A, normal human adult hemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; NMR, nuclear magnetic resonance; ppm, parts per million; HDO, residual H_2O in D_2O medium; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] A great majority of the investigations of the Bohr effect have been carried out with the carbonmonoxy rather than the oxy form of Hb. This choice is dictated by the fact that HbCO A is more stable than its oxy analogue (against acid and alkaline denaturation, oxidation to met-Hb, etc.). According to Antonini et al. (1963), there is no difference between HbO_2 and HbCO in the amount of H^+ ions bound by Hb at any pH studied.

0006-2960/87/0426-6299$01.50/0

(1970) proposed a molecular mechanism for the Bohr effect in which the α-amino of α1Val and the imidazole of β146His form "salt bridges" with negatively charged groups in deoxy-Hb, thereby increasing their affinity for H^+ ions. On oxygenation, these salt bridges are broken, the pK values of the Bohr groups revert to normal, and protons are released. The involvement of α1Val was first demonstrated by carbamylation of its amino group by cyanate, which decreased the alkaline Bohr effect (Kilmartin & Rossi-Bernardi, 1969). It is now known that α1Val binds an anion (chloride) and thus forms a salt bridge with the guanidinium group of α141Arg of the other α chain in the deoxy form, but not in HbO_2 (Arnone et al., 1977; O'Donnell et al., 1979). In 0.1 M Cl^- at 25 °C, the pK of α1Val is 8.0 in the deoxy form and 7.25 in the CO form. This corresponds to a 20–30% contribution to the Bohr effect from this group under these conditions (Garner et al., 1975; Matthew et al., 1977; van Beek et al., 1978; O'Donnell et al., 1979).

β146His forms a salt bridge with β94Asp in deoxy-Hb crystals but not in oxy-like met-Hb crystals (Perutz, 1970). In all the Hb molecules in which this histidyl residue is altered, either by enzymatic or chemical modification or by replacement by mutation, the alkaline Bohr effect is reduced compared to that of Hb A [for example, see Perutz et al. (1980), Kilmartin et al. (1980), and Matsukawa et al. (1984)]. However, the amount of this reduction depends on the nature of the alteration or of the replacing amino acid residue as well as on the experimental conditions. For example, the β146His → Asp substitution (Hb Hiroshima), β146His → Pro substitution (Hb York), and β146His → Leu substitution (Hb Cowtown) result in reduction of the alkaline Bohr effect by about 50% (Imai, 1968; Bare et al., 1976; Shih et al., 1984). On the other hand, the β146His → Arg substitution in Hb Cochin–Port Royal and the substitution of His for β94Asp (the partner residue of β146His in the salt bridge) in Hb Barcelona lower the alkaline Bohr effect by only 20–30% (Wajcman et al., 1975, 1982; Phillips et al., 1983). When the β146His residues are simply removed by carboxypeptidase B digestion as in Hb des(Hisβ146), the corresponding reduction in the alkaline Bohr effect varies from 40% to 60% depending upon the concentration of Cl^- ions (Kilmartin & Wootton, 1970; Kilmartin et al., 1980).

Saroff (1972) was first to point out that the identification of the Bohr groups using mutant or chemically modified Hbs requires a complete analysis of the pH dependence of the number of H^+ ions released upon oxygenation. This is due to the fact that replacements or chemical modifications of single amino acids can induce long-range conformational effects in the Hb molecule and thus can affect the contributions of other groups to the Bohr effect. For example, in the case of Hb des(Hisβ146), the reduction in the Bohr effect cannot be ascribed to the simple removal of a Bohr group (namely, β146His), but the pK values of other groups can be affected (Saroff, 1972; Matsukawa et al., 1984). Our recent results from Hbs containing single-site structural perturbations have also suggested that additional mechanisms must be involved in the alterations of the Bohr effect in these modified Hbs (Russu et al., 1982; Ho & Russu, 1985). Thus, the interpretation of effects observed in chemically modified or mutant Hbs is open to doubt.

Deuterium or Tritium Exchange for the C2 Protons of Histidyl Residues of Hb A

Ohe et al. (1974) developed a hydrogen-exchange method for the determination of the pK values of individual histidyl residues in intact proteins. This method is based on the measurement of the pseudo-first-order rate constant for the exchange of the C2 proton of histidine by ^2H or ^3H as a function of pH.[3] The protein is then cleaved by proteases, and the specific histidyl residues are identified in the cleaved peptides. The hydrogen-exchange method has been applied to the Bohr effect of Hb A in the presence of 0.1 M Cl^- by three laboratories (Nishikura, 1978; Ohe & Kajita, 1980; Matsukawa et al., 1984), and the reported results on the pK values are conflicting. Nishikura (1978) reported the pK of α122His in deoxy-Hb A as 6.1 and that in HbCO A as 6.6. However, Ohe and Kajita (1980) found that α122His is not titratable. Matsukawa et al. (1984) reported that the pK value of β146His changed from 8.0 in the deoxy-Hb A to 6.5 in HbCO A and concluded that β146His can contribute about 75% of the alkaline Bohr effect at pH 7.5. They also believe that β143His contributes 50% of the acid Bohr effect. Ohe and Kajita (1980) reported that α20His, α89His, and β146His are histidyl residues responsible for the alkaline Bohr effect and that α89His and β146His play a major role because of the large change in their pK values in going from the deoxy to the CO form (ΔpK = 1.6 and 1.1, respectively). It should be pointed out that, according to the pK values measured by Ohe and Kajita (1980), the sum of protons released by α20His, α89His, β143His, and β146His plus α1Val in going from the deoxy to the CO form of Hb A greatly exceeds the total Bohr protons calculated on the basis of the results of Antonini et al. (1965).

The disagreement in the pK values determined by the ^2H or ^3H exchange reaction method could be due to both technical and conceptual difficulties in using this methodology. First, the exchange reaction has to be carried out under experimental conditions rather severe for the Hb molecule, i.e., in the presence of a reducing agent (sodium dithionite or sodium hydrosulfite) to prevent the formation of met-Hb and for a period of 24–100 h at ~37 °C over a pH range from 4.9 to 9.5 (Ohe & Kajita, 1980; Nishikura, 1978; Matsukawa et al., 1984). Second, the experimental conditions used in these laboratories for preparation of peptides could have been different, and thus, the back-exchange rates of ^2H or ^3H might not have been identical. Conceptually, one also needs to be concerned that the surface histidyl residues in the Hb molecule may have different degrees of exposure to the solvent, which could complicate the determination of the pK values from ^1H to ^2H (or ^3H) exchange data. Thus, the pK values of histidyl residues of Hb A determined by the exchange reaction need further investigation.

Electrostatic Model of the Bohr Effect

A modified Tanford–Kirkwood discrete charge theory has been applied to model the electrostatic interactions and the single-site H^+ equilibria in Hb A by Gurd and co-workers (Matthew et al., 1979a,b, 1982, 1985). This treatment deals with the complete array of point charges in the Hb molecule and includes all ionizable proton binding groups and the bound anions. The free energy of the electrostatic interactions between such protein charges is corrected according to the fractional solvent accessibility of each group as determined from the crystal structure (Lee & Richards, 1971). For a detailed discussion of the model, refer to Matthew et al. (1985). The electrostatic calculations were carried out for the

[3] The data on the Bohr effect derived from the hydrogen–deuterium exchange reaction and from ^1H NMR studies were obtained in D_2O rather than in H_2O. The rationale for doing so is given in Ohe et al. (1974) and Russu et al. (1980).

Table I: pK Values of Histidyl Residues in Hb A in Deoxy and CO Forms at 27–29 °C

		pK			
resonance	assignment[a]	0.1 M Bis-Tris/ 0.1 M Tris[b]	0.1 M Bis-Tris/ 0.18 M Cl^-[c]	0.1 M phosphate[d]	0.1 M Bis-Tris + 2,3-DPG[e]
			Deoxy-Hb A		
1	β97His	8.13 ± 0.06	7.95 ± 0.04	7.81 ± 0.02	7.88 ± 0.03
2		7.29 ± 0.02	7.48 ± 0.03	7.28 ± 0.02	7.18 ± 0.01
3	β146His	7.98 ± 0.05	7.94 ± 0.02	7.82 ± 0.03	8.12 ± 0.04
4		7.21 ± 0.03	7.40 ± 0.04	7.25 ± 0.02	7.09 ± 0.01
4′		7.21 ± 0.03	7.47 ± 0.03	7.19 ± 0.02	7.05 ± 0.02
5		7.92 ± 0.05	7.58 ± 0.03	7.57 ± 0.03	7.71 ± 0.03
6		7.10 ± 0.03	7.12 ± 0.02	7.16 ± 0.02	6.98 ± 0.02
7	β116His and	7.02 ± 0.08	7.07 ± 0.04	6.84 ± 0.04	7.04 ± 0.02
8	β117His	6.63 ± 0.09	6.64 ± 0.09	6.58 ± 0.13	6.30 ± 0.18
9		6.61 ± 0.07	6.60 ± 0.20	6.88 ± 0.05	6.80 ± 0.02
10	β2His	6.32 ± 0.05	6.43 ± 0.03	7.01 ± 0.03	7.13 ± 0.05
			Carbonmonoxy-Hb A		
A		7.90 ± 0.03	7.88 ± 0.05	7.81 ± 0.01	7.90 ± 0.02
B		7.42 ± 0.02	7.43 ± 0.02	7.35 ± 0.04	7.33 ± 0.02
C	(β146His)[f]	7.85 ± 0.04	7.74 ± 0.02	7.56 ± 0.03	8.01 ± 0.09
D		7.68 ± 0.03	7.67 ± 0.05	7.78 ± 0.14	7.74 ± 0.03
E		7.14 ± 0.03	7.21 ± 0.02	7.28 ± 0.03	7.04 ± 0.02
F		6.99 ± 0.03	6.98 ± 0.02	7.12 ± 0.02	7.01 ± 0.01
G	β2His	6.49 ± 0.03	6.28 ± 0.09	6.82 ± 0.01	6.68 ± 0.02
H		6.20 ± 0.05	not measured	not measured	5.84 ± 0.18
I		6.59 ± 0.05	6.49 ± 0.03	6.60 ± 0.03	5.60 ± 0.41
J	β116His and	6.78 ± 0.03	6.47 ± 0.05	6.68 ± 0.01	6.45 ± 0.09
K	β117His	6.22 ± 0.18	6.40 ± 0.05	6.58 ± 0.04	6.51 ± 0.04
L	β143His	too low to measure	too low to measure	too low to measure	broadened beyond detection

[a] The assignments of the C2-proton resonances of the histidyl residues of Hb A were taken from Russu et al. (1980, 1982, 1984) and Ho and Russu (1985). [b] At 27 °C; chloride ion concentration from 0.005 to 0.06 M; taken from Tables IV and V of Russu et al. (1982). [c] At 29 °C; G. Kellogg, I. M. Russu, and C. Ho, unpublished results. [d] At 29 °C; A. K.-L. C. Lin, I. M. Russu, and C. Ho, unpublished results. [e] At 27 °C (1:1 molar ratio of 2,3-DPG to Hb A); K. Bupp, W.-G. Wu, I. M. Russu, and C. Ho, unpublished results. [f] This assignment is controversial in 0.1 M Bis-Tris or 0.1 M Tris buffer at low anion concentration. For details, see the text.

deoxy-Hb A structure at 2.5-Å resolution (Fermi, 1975), for the HbO_2 A structure at 2.1 Å (Shanaan, 1983), and for the structure of HbCO A at 2.7 Å (Baldwin, 1980). The H^+ titration curves predicted on the basis of these structures differ by no more than one charge from those measured experimentally (Matthew et al., 1985). According to this electrostatic model, the Bohr effect does not result from a few, well-defined rearrangements in salt-bridged amino acid residues but is a thermodynamic consequence of the unique charge distributions in the two quaternary structures of the Hb molecule. The major limitations of this electrostatic model are the following: (i) there is a lack of detailed knowledge about the protein–solvent interfaces, and (ii) it does not include all the local conformations that may be assumed by surface amino acid residues in solution [for example, see Matthew (1985)]. In addition, there could also be pH-induced conformational changes in the protein molecule, which make the electrostatic modeling very difficult.

Proton NMR Investigation

High-resolution proton nuclear magnetic resonance (NMR) spectroscopy is ideally suited to investigate the molecular basis of the Bohr effect. The C2- and C4-proton resonances of the histidyl residues of Hb A are well separated from those of other protons and usually occur in the aromatic proton resonance region (Figure 1). The C2-proton resonances of 22 histidyl residues (or 11 per $\alpha\beta$ dimer) can be resolved and titrated individually by ^1H NMR in both deoxy and CO forms (Russu et al., 1982). The corresponding pK values are given in Table I. The number of H^+ ions released per Hb as derived from these pK values, and with the assumption that the same 22 histidyl residues were observed in the deoxy form as in the CO form, is comparable to that observed experimentally (Russu et al., 1982).

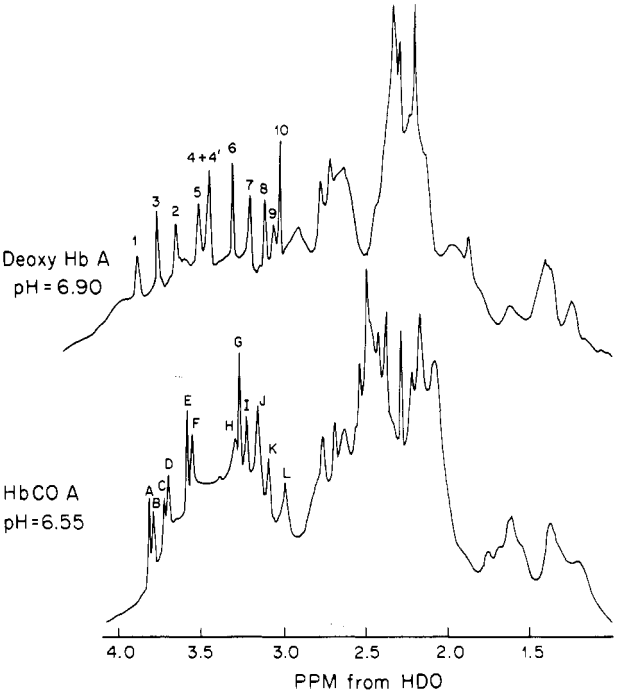

FIGURE 1: 300-MHz ^1H NMR spectra of 10% Hb A in 0.1 M Bis-Tris in D_2O at 29 °C. The proton chemical shift of HDO is 4.73 ppm downfield from that of the methyl protons of a water-soluble standard, the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 29 °C.

A detailed understanding of the molecular mechanism of the Bohr effect by ^1H NMR spectroscopy requires the identification of each histidyl residue of Hb in the NMR spectra. By a careful comparison of the ^1H NMR spectra of Hb A with those of histidine-mutant and histidine-modified Hbs, we have assigned several C2-proton resonances to specific histidyl

residues in Hb A in both deoxy and CO forms (Kilmartin et al., 1973; Russu et al., 1980, 1982, 1984; Ho & Russu, 1981, 1985; Russu & Ho, 1986). These assignments are given in Table I.

Using des(Hisβ146)Hb, we have first identified the C2- and C4-proton resonances of β146His in both deoxy and CO forms (Kilmartin et al., 1973; Russu et al., 1980, 1982). On the basis of these assignments, we have found that the contribution of β146His to the Bohr effect depends upon experimental conditions. In the presence of 0.2 M phosphate plus 0.2 M NaCl, the pK value of β146His decreases upon ligation from 8.0 to 7.1 at 30 °C, and thus β146His can account for ~50% of the alkaline Bohr effect (Kilmartin et al., 1973). In 0.1 M Bis-Tris and/or 0.1 M Tris buffer in the presence of 0.005–0.06 M Cl^- (the so-called "stripped" conditions) at 27 °C, the pK values of β146His are 8.0 and 7.85 in the deoxy and CO forms of Hb A, respectively (Russu et al., 1980, 1982), and the contribution of β146His to the alkaline Bohr effect is quite small, namely, less than 5%. Under the same experimental conditions, the total Bohr effect is, however, preserved (Russu et al., 1982), suggesting that several other histidyl residues contribute to the effect. The conclusion from these results is that the detailed molecular mechanism of the Bohr effect depends on solvent composition (Russu et al., 1980, 1982).

This conclusion on the role of β146His in the Bohr effect was questioned by Perutz and co-workers on the basis of biochemical, structural, and ^{1}H NMR studies of mutant and enzymatically or chemically modified Hbs (Perutz et al., 1980, 1985a,b; Kilmartin et al., 1980). In two recent studies, Perutz and co-workers compared the aromatic ^{1}H resonances of HbCO A with those of HbCO Cowtown (β146His → Leu), HbCO des-His(β146), HbCO Wood (β97His → Leu), HbCO Malmö (β97His → Gln), HbCO Abbruzzo (β143His → Arg), HbCO Barcelona (β94Asp → His), and HbCO Fort de France (α45His → Arg) in 0.2 M HEPES or 0.1 M Bis-Tris in the absence and presence of 0.1 M Cl^- at 30 °C (Perutz et al., 1985a,b). They concluded that resonance C (Figure 1) should be assigned to β97His (and not to β146His as proposed by Ho and co-workers). Instead, resonance H was proposed as originating from the β146His C2 proton. According to these assignments, the pK value of β146His in HbCO A is 6.2 and that for β97His in HbCO A should be 7.85. In the deoxy form, the corresponding pK values are 8.0 for β146His and 8.1 for β97His (resonances 3 and 1 in Figure 1) (Russu et al., 1980, 1982; Perutz et al., 1985a). The large change in the pK value of β146His in going from the deoxy to the CO state (ΔpK = 1.8) found by Perutz et al. (1985a,b) implied that β146His should contribute about 75% of the Bohr effect and the Bohr effect would exceed that measured experimentally (Antonini et al., 1965).

Our recent NMR investigation of similar mutant Hbs (Russu & Ho, 1986) has clearly shown that resonance H of HbCO A cannot be the C2 proton of β146His and that the assignment proposed by Perutz et al. (1985a,b) was due to experimental artifacts in the sample preparations (such as contamination with oxy- and/or met-Hb). Following our study, Perutz and co-workers (Shih et al., 1987) have reached the same conclusion, namely, that resonance H cannot be due to β146His. The discrepancy between this result (Shih et al., 1987) and those observed previously (Perutz et al., 1985a,b) is, however, attributed to the method used to exchange the Hb solution into a deuteriated solvent, i.e., by pressure filtration or by dialysis (Shih et al., 1987).

Resonance C [assigned to the C2 proton of β146His by Russu et al. (1980, 1982)] is missing from the ^{1}H NMR spectra of Hb molecules containing structural perturbations at β146, β143, β97, and β94 sites such as HbCO des-His-(β146), HbCO Wood (β97His → Leu), HbCO Malmö (β97His → Gln), HbCO Barcelona (β94Asp → His), and HbCO Abbruzzo (β143His → Arg) (Perutz et al., 1985a,b; Russu & Ho, 1986). However, this resonance is present in Hb Cowtown. These results cannot be used at present to assign this resonance to β97His as proposed by Perutz et al. (1985a,b) for the following reasons. First, there are substantial spectral alterations among HbCO A, HbCO Wood, and HbCO Malmö [see Figures 6 and 7 of Russu and Ho (1986)], and thus, the absence of resonance C cannot be solely attributed to the absence of β97His residues in the two mutant Hbs. Second, in Hb Cowtown, the β146His → Leu substitution induces changes in several surface histidyl residues as well as in the conformation of amino acid residues situated in the heme pockets of the α and β chains (Russu & Ho, 1986). Thus, HbCO Cowtown is not a suitable mutant to use to assign the proton resonance of the β146His in HbCO A. This suggests that different parts of the Hb molecule are interactive [for example, see Ho and Russu (1985)]. At present, the mechanisms responsible for the absence or the presence of resonance C in mutant or enzymatically modified Hbs remain an open question.

In a recent study, Craescu et al. (1986) have proposed assignments for the C2-proton resonances of the following histidyl residues in Hb A in both deoxy and oxy forms: α20, α50, α72, α89 (deoxy form only), β112, β2, β77, β97 (deoxy form only), and β117 (deoxy form only). These assignments are questionable on the basis of the following points. First, the spectral comparisons between Hb A and the mutant Hbs are very difficult due to substantial spectral perturbations in the mutant Hbs and also due to the manner in which Craescu et al. (1986) processed their ^{1}H NMR data, which affected the intensities of the resonances of interest (for example, see resonance 10 in their Figure 4). Second, they used the same spectral labeling system for HbO_2 as that used by Russu et al. (1980) for HbCO A, but they ignored the substantial differences in the NMR spectra between HbCO A and HbO_2 A (Russu & Ho, 1986). Thus, they should not use the same spectral labeling system for HbO_2 and HbCO even though the Bohr effect due to the binding of CO to Hb A is the same as that due to O_2 (Antonini et al., 1963).[2] Third, on the basis of their resonance assignments and the assignment-independent pK values of histidyl residues (Russu et al., 1982), the following histidyl residues would make significant contributions to the alkaline Bohr effect (based on $\Delta pK = pK_{deoxy} - pK_{oxy}$): α50 (ΔpK = 0.22), α72 (ΔpK = −0.13), α112 (ΔpK = 0.24), β2 (ΔpK = 0.53), and β77 (ΔpK = −0.69; i.e., β77His residues would make a *negative* contribution of ~40% to the Bohr effect). Fourth, the results predict that β117His residues (assigned to resonance 10 in Figure 1) are the strongest binding sites for inorganic phosphate and 2,3-DPG in deoxy-Hb A (see also Table I). All these predictions are inconsistent with known structural properties of Hb. Thus, the spectral assignments for the histidyl residues proposed by Craescu et al. (1986) require further investigation.

In conclusion, the assignment of the proton NMR resonances of histidyl residues in Hb A using mutant and chemically modified Hbs is limited by the possible long-range conformational effects of substitutions and/or chemical modifications of single amino acid residues in the molecule. Different parts of the Hb molecule are clearly interactive. These long-range conformational interactions should be considered when structural changes at individual sites are cor-

related with spectral perturbations or global functional properties.

Effects of Anions on the Bohr Effect of Hemoglobin

Extensive experimental evidence over the past two decades has demonstrated that a large fraction of the Bohr effect of Hb originates from the heterotropic interactions between Hb A and anions. These effects can be explained as being due to a difference in the anion binding affinities between the deoxy and ligated states of Hb A (van Beek et al., 1979; Bucci & Fronticelli, 1985). ^{35}Cl NMR has confirmed that deoxy-Hb A has a higher affinity for Cl^- ions than ligated Hb (Chiancone et al., 1972, 1975; Bull et al., 1973). An analysis of the Cl^- dependence of the Bohr effect has revealed that, at physiological pH and ionic strength, about half of the Bohr protons result from this difference in the Cl^- affinity between deoxy- and oxy-Hb. Similar thermodynamic models have been used to explain the enhancements in the alkaline Bohr effect induced by inorganic and organic phosphates (Riggs, 1971; de Bruin et al., 1974a; Bonaventura & Bonaventura, 1978).

We have recently proposed that anions can exert their influence on the Bohr effect by also affecting the conformational changes occurring in the Hb molecule upon ligation (Russu et al., 1980, 1982). On the basis of ^1H NMR results, we have suggested that the breaking of the β146His–β94Asp salt bridge upon oxygenation occurs only in the presence of appropriate concentrations of chloride and/or phosphate ions. As a result, the contribution of β146His to the Bohr effect is ~5% in stripped conditions and ~50% in the presence of 0.2 M phosphate plus 0.2 M Cl^- ions (Kilmartin et al., 1973; Russu et al., 1980).

Very recently, Shih and Perutz (1987) addressed the same mechanism by measuring the alkaline Bohr effect and the individual Adair constants of Hb A and Hb Cowtown (β146His → Leu) under various solvent conditions.[4] Their main findings are as follows: β146His contributes nearly all the alkaline Bohr effect (94%) in 0.1 M HEPES buffer (Cl^- free), ~57% in 0.05 M Bis-Tris buffer (with minimum Cl^- concentration), ~52% in 0.05 M Bis-Tris (with 0.1 M Cl^-), and close to zero in 0.05 M Bis-Tris in the presence of 0.1 M chloride plus 2 mM IHP. Shih and Perutz (1987) have proposed that IHP inhibits the contribution of the β146His to the alkaline Bohr effect because it "keeps its salt bridge intact even in the R structure". This suggestion is not supported by the ^1H NMR results of Kilmartin et al. (1978), who found that, in the presence of 20–40 mM chloride and a twofold excess of IHP (over the Hb concentration) at 30 °C, the pK values of β146His are 8.2 and 7.1 for the deoxy and CO forms of Hb A. Hence, both mechanisms involving the β146His in the anion dependence of the Bohr effect of Hb require further investigation.

At present, one of the most important questions for the understanding of the Bohr effect remains the location and characterization of the anion binding sites in the Hb molecule in both deoxy and ligated states. The location of a strong Cl^- binding site in deoxy-Hb A is believed to be at the salt bridge between the α-amino group of α1Val and the guanidinium of α141Arg of the neighboring α chain (Arnone et al., 1977; O'Donnell et al., 1979). In the oxy form of Hb A, this salt bridge is broken and the Cl^- ion is released. Perutz et al. (1980) have proposed that β82Lys could be responsible for 20–30% of the Bohr effect due to its weak binding of Cl^- ions. However, this contribution is unlikely because the normal pK for lysine is ~10.5, a value too high to make a significant contribution to the Bohr effect. Several additional binding sites for Cl^- ions in deoxy-Hb have been suggested by the electrostatic calculations of Matthew et al. (1982). The magnitude of the alkaline Bohr effect at 0.1 M ionic strength could be accounted for theoretically by assuming binding of a Cl^- ion at α1Val and at any of the following sites: β117His, between β2His and β143His, or between β1Val and β82Lys. Binding sites for 2,3-DPG and IHP have been identified in deoxy-Hb A by X-ray diffraction (Arnone, 1972; Arnone & Perutz, 1974).

We have carried out a ^1H NMR investigation of the effects of Cl^- and inorganic and organic phosphate ions upon the pK values of surface histidyl residues of deoxy-Hb A and HbCO A in D_2O at 29 °C and at constant ionic strength (I. M. Russu, A. K.-L. C. Lin, S. S. Wu, K. Bupp, G. Kellogg, and C. Ho, unpublished results). The pK values of several surface histidyl residues are increased by the presence of either Cl^- or phosphate ions, consistent with the binding of the anions to these sites (Table I). However, some of the binding sites for Cl^- ions are different from those for inorganic phosphate ions. The binding site for 2,3-DPG detected by NMR clearly involves the β2His residues, in agreement with X-ray diffraction (Arnone, 1972) and biochemical evidence. The β2His residues are also found to be involved in the binding of inorganic phosphate ions to both deoxy-Hb and HbCO A. A more detailed discussion of this subject will be published elsewhere.

Conclusions

The main conclusion based on the available experimental evidence is that the detailed molecular mechanism for the alkaline Bohr effect of Hb A depends on the solvent composition and on the nature of the anions.

Both α1Val and β146His play important roles in the Bohr effect in the presence of Cl^- and/or inorganic phosphate. In the absence or at low concentrations of Cl^- and/or inorganic phosphate ions, there is disagreement as to whether or not β146His would make a significant contribution to the alkaline Bohr effect. This controversy is due to the lack of definitive assignment of the C2-proton resonance due to β146His in HbCO A. Further investigation in this direction is clearly needed.

β2His in both deoxy and CO forms of Hb A is a strong binding site for both inorganic phosphate and 2,3-DPG. Thus, this residue plays an important role in the Bohr effect of Hb A in the presence of inorganic and organic phosphate ions. The assignment of the C2-proton resonances of β2His (resonances 10 and G in Figure 1) can be regarded as being definitive.

Several other proton resonances of histidyl residues in Hb A are affected by Cl^- and/or phosphate ions (for example, resonances 2, 4, and 4′ in deoxy-Hb and D, E, and F in ligated Hb A). Thus, these histidyl residues are anion binding sites and contribute to the anion-dependent Bohr effect. The assignment of these proton resonances to specific histidyl residues awaits further investigation.

Our current knowledge of the acid Bohr effect of Hb A is very limited. It has been proposed that β143His residues contribute about 50% of the acid Bohr effect because its pK values are lowered by its two neighboring lysyl residues (β82 and β144) (Perutz et al., 1980). Our ^1H NMR results show

[4] Very recently, Nagai and co-workers have "engineered" a mutant Hb, Hb Bulltown (β146His → Gln), and have investigated the X-ray structure for the deoxy form and the Bohr effect (K. Nagai, personal communication). Their results indicate that the deoxy structure of deoxy-Hb Bulltown is very similar to that of deoxy-Hb A at 2.6-Å resolution and that the results on the Bohr effect of Hb Bulltown are analogous to those of Hb Cowtown.

that the C2-proton resonance of β143His in HbCO A in 0.1 M Bis-Tris buffer has a pK value of <6.3. Due to technical difficulties at lower pH values, the precise pK value of this residue cannot be measured.

In conclusion, due to the extensive efforts of a large number of investigators during the past three decades, we now have a much better understanding of the structure–function relationship in the Hb molecule, but the detailed molecular mechanism for the Bohr effect is not yet elucidated. This is especially true for the acid Bohr effect. It is obvious that new techniques and approaches are needed to assign and characterize various amino acid residues that are involved in the Bohr effect of Hb A under various experimental conditions. The results presented in this review clearly show that Hb is an interactive molecule in which perturbations (either by ligand or by amino acid substitution) at one site can be communicated to other sites. The Bohr effect is an excellent model for investigating the molecular basis for heterotropic interactions in allosteric proteins, in general.

ACKNOWLEDGMENTS

C.H. extends his appreciation to Professor George K. Radda for providing a stimulating environment to spend a sabbatical and to write this review in the Department of Biochemistry at the University of Oxford. We thank Professor Robert J. P. Williams, Dr. James B. Matthew, and Dr. E. Ann Pratt for helpful discussions.

REFERENCES

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, Elsevier/North-Holland, London.

Antonini, E., Wyman, J., Rossi-Fanelli, A., & Caputo, A. (1962) *J. Biol. Chem. 237*, 2773.

Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronticelli, C., & Rossi-Fanelli, A. (1963) *J. Biol. Chem. 238*, 2950.

Antonini, E., Wyman, J., Brunori, M., Fronticelli, C., Bucci, E., & Rossi-Fanelli, A. (1965) *J. Biol. Chem. 240*, 1096.

Antonini, E., Amiconi, G., & Brunori, M. (1972) in *Oxygen Affinity of Hemoglobin and Red Cell Acid–Base Status* (Astrap, P., & Roth, M., Eds.) pp 121–129, Academic, New York.

Arnone, A. (1972) *Nature (London) 237*, 146.

Arnone, A., & Perutz, M. F. (1974) *Nature (London) 249*, 34.

Arnone, A., Benesch, R. E., & Benesch, R. (1977) *J. Mol. Biol. 115*, 627.

Baldwin, J. M. (1980) *J. Mol. Biol. 136*, 103.

Bare, G. H., Bromberg, P. A., Alben, J. O., Brimhall, B., Jones, R. T., Mintz, S., & Rother, I. (1976) *Nature (London) 259*, 155.

Benesch, R. E., Benesch, R., & Yu, C. I. (1969) *Biochemistry 8*, 2567.

Benesch, R. E., Benesch, R., Kwang, S., & McCord, J. M. (1986) *J. Mol. Biol. 190*, 481.

Bonaventura, C., & Bonaventura, J. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 647–663, Academic, New York.

Bucci, E., & Fronticelli, C. (1985) *Biochemistry 24*, 371.

Bull, T. E., Andrasko, J., Chiancone, E., & Forsen, S. (1973) *J. Mol. Biol. 73*, 251.

Chiancone, E., Norne, J. E., Forsen, S., Antonini, E., & Wyman, J. (1972) *J. Mol. Biol. 70*, 675.

Chiancone, E., Norne, J. E., Forsen, S., Bonaventura, J., Brunori, M., Antonini, E., & Wyman, J. (1975) *Eur. J. Biochem. 55*, 385.

Craescu, C. T., Schaeffer, C., Mispelter, J., Garin, J., & Rosa, J. (1986) *J. Biol. Chem. 261*, 7894.

De Bruin, S. H., Janssen, L. H. M., & van Os, G. A. J. (1973) *Biochem. Biophys. Res. Commun. 55*, 193.

De Bruin, S. H., Rollema, H. S., Janssen, L. H. M., & van Os, G. A. J. (1974a) *Biochem. Biophys. Res. Commun. 58*, 204.

De Bruin, S. H., Rollema, H. S., Janssen, L. H. M., & van Os, G. A. J. (1974b) *Biochem. Biophys. Res. Commun. 58*, 210.

Edsall, J. T. (1972) *J. Hist. Biol. 5*, 205.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237.

Fronticelli, C., Bucci, E., & Orth, C. (1984) *J. Biol. Chem. 259*, 10841.

Garner, M. H., Bogardt, R. A., Jr., & Gurd, F. R. N. (1975) *J. Biol. Chem. 250*, 4398.

Ho, C., & Russu, I. M. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 179–193, Academic, New York.

Ho, C., & Russu, I. M. (1981) *Methods Enzymol. 76*, 275.

Ho, C., & Russu, I. M. (1985) in *New Methodologies in Studies of Protein Configuration* (Wu, T. T., Ed.) pp 1–35, Van Nostrand–Rheinhold, Princeton, NJ.

Imai, K. (1968) *Arch. Biochem. Biophys. 127*, 543.

Kilmartin, J. V., & Rossi-Bernardi, L. (1969) *Nature (London) 222*, 1243.

Kilmartin, J. V., & Wootton, J. F. (1970) *Nature (London) 228*, 766.

Kilmartin, J. V., & Rossi-Bernardi, L. (1973) *Physiol. Rev. 53*, 836.

Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246.

Kilmartin, J. V., Anderson, N. L., & Ogawa, S. (1978) *J. Mol. Biol. 123*, 71.

Kilmartin, J. V., Fogg, J. H., & Perutz, M. F. (1980) *Biochemistry 19*, 3189.

Lee, B. K., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379.

Matsukawa, S., Itatani, Y., Mawatari, K., Shimokawa, Y., & Yoneyama, Y. (1984) *J. Biol. Chem. 259*, 11479.

Matthew, J. B. (1985) *Annu. Rev. Biophys. Chem. 14*, 387.

Matthew, J. B., Morrow, J. S., Wittebort, R. J., & Gurd, F. R. N. (1977) *J. Biol. Chem. 252*, 2234.

Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979a) *Biochemistry 18*, 1919.

Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979b) *Biochemistry 18*, 1928.

Matthew, J. B., Friend, S. H., & Gurd, F. R. N. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Eaton, W. A., Collman, J. P., Gibson, Q. H., Leigh, J. S., Jr., Moffat, K., Margoliash, E., & Scheidt, W. R., Eds.) pp 231–241, Elsevier/North-Holland, New York.

Matthew, J. B., Gurd, F. R. N., Garcia-Moreno, E. B., Flanagan, M. A., March, K. L., & Shire, S. J. (1985) *CRC Crit. Rev. Biochem. 18*, 91.

Nishikura, K. (1978) *Biochem. J. 173*, 651.

O'Donnell, S., Mandaro, R., Schuster, T. M., & Arnone, A. (1979) *J. Biol. Chem. 254*, 12204.

Ohe, M., & Kajita, A. (1980) *Biochemistry 19*, 4443.

Ohe, M., Matsuo, H., Sakiyama, F., & Narita, K. (1974) *J. Biochem. (Tokyo) 75*, 1197.

Perutz, M. F. (1970) *Nature (London) 228*, 726.

Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J. G., & Rollema, H. S. (1980) *J. Mol. Biol. 138*, 649.
Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H., & Shih, D. T.-b. (1985a) *J. Mol. Biol. 183*, 491.
Perutz, M. F., Gronenborn, A. M., Clore, G. M., Shih, D. T.-b., & Craescu, C. T. (1985b) *J. Mol. Biol. 186*, 471.
Phillips, S. E. V., Perutz, M. F., Poyart, C., & Wajcman, H. (1983) *J. Mol. Biol. 164*, 477.
Riggs, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2062.
Rollema, H. S., de Bruin, S. H., Janssen, L. H. M., & van Os, G. A. J. (1975) *J. Biol. Chem. 250*, 1333.
Russu, I. M., & Ho, C. (1986) *Biochemistry 25*, 1706.
Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043.
Russu, I. M., Ho, N. T., & Ho, C. (1982) *Biochemistry 21*, 5031.
Russu, I. M., Lin, A. K.-L. C., Ferro-Dosch, S., & Ho, C. (1984) *Biochim. Biophys. Acta 785*, 123.
Saroff, H. A. (1972) *Physiol. Chem. Phys. 4*, 23.
Shanaan, B. (1983) *J. Mol. Biol. 171*, 31.
Shih, D. T.-b., & Perutz, M. F. (1987) *J. Mol. Biol. 195*, 419.
Shih, D. T.-b., Jones, R. T., Bonaventura, J., Bonaventura, C., & Schneider, R. G. (1984) *J. Biol. Chem. 259*, 967.
Shih, D. T.-b., Perutz, M. F., Gronenborn, A. M., & Clore, G. M. (1987) *J. Mol. Biol. 195*, 453.
Van Beek, G. G. M., & de Bruin, S. H. (1980) *Eur. J. Biochem. 105*, 353.
Van Beek, G. G. M., Zuiderweg, E. R. P., & de Bruin, S. H. (1978) *Eur. J. Biochem. 92*, 309.
Van Beek, G. G. M., Zuiderweg, E. R. P., & de Bruin, S. H. (1979) *Eur. J. Biochem. 99*, 379.
Wajcman, H., Kilmartin, J. V., Najman, A., & Labie, D. (1975) *Biochim. Biophys. Acta 400*, 354.
Wajcman, H., Aguilar, J. L., Bascompte, I., Labile, D., Poyart, C., & Bohn, B. (1982) *J. Mol. Biol. 156*, 185.
Wyman, J. (1948) *Adv. Protein Chem. 4*, 407.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223.

Accelerated Publications

How Membrane Chain Melting Properties Are Regulated by the Polar Surface of the Lipid Bilayer†

Gregor Cevc

Laboratorium für experimentelle Urologie, Universitätsklinikum Essen, D-4300 Essen, FRG, and Max-Planck-Institut für biophysikalische Chemie, Am Fassberg, D-3400 Göttingen, FRG

Received May 13, 1987; Revised Manuscript Received June 19, 1987

ABSTRACT: The principle of regulation of various membrane properties by the hydrocarbon membrane interior is now well understood. The mechanism by which the interfacial membrane region including aqueous solution affects the state of the lipid bilayer matrix, however, is as yet unclear, despite its great biological and physiological significance. Data and analysis presented in this paper show that apart from the lipid chain type, length, and degree of unsaturation the main factors determining the characteristics of lipid membranes are surface polarity and interfacial hydration. These incorporate the effects of head group dipole and multipole moments as well as the head group ability for hydrogen bonding and can account for most of the changes in the physicochemical membrane state caused by the lipid head group structure, bulk pH value, salt content, solute adsorption, etc. The effects of membrane potential are much less, only 10–30% of the former. Variations in hydration thus not only govern the short- and medium-range intermolecular and intermembrane interactions but also provide a fast and energetically inexpensive regulatory mechanism for lipid membranes to adapt their characteristics, at least locally or transiently, to new requirements.

Phospholipids are one of the major constituents of biological membranes. They assemble into bilayers, which provide cells with permeability barriers and with matrices for the insertion of the nonlipid membrane components. The biochemical and biological functions of all membrane components, such as proteins, are therefore influenced by the local properties, or accumulation of lipids near such components [cf. Jost and Griffith (1982)], and varying the type or the physicochemical state of these lipids can thus contribute to controlling the state and functions of a given membrane region. The role of the apolar bilayer interior in this has long been recognized (Marčelja, 1973; Pink, 1982), but not even an elementary understanding of the principles existed to date that would allow an analysis of the effect of the polar membrane region, including the ionic solution, on the membrane characteristics.

Attempts have been made to explain the effect of lipid ionization on the bilayer chain melting phase transition temperature by using classical electrostatic double layer theory [cf. Träuble et al. (1976)]; however, it is now rather certain that only a fraction of the ionization-induced chain melting phase transition shift is indeed of simple electrostatic origin (Cevc et al., 1986; Cevc & Marsh, 1987; also further discussion). To interpret the effects of chemical head group modification, e.g., the consequences of methylation, on the membrane phase behavior, the differences in electrostatic moments and/or bulkiness of the lipid head groups have been

† Supported by the Deutsche Forschungsgemeinschaft (SFB 102 D14).

0006-2960/87/0426-6305$01.50/0