Anion Bohr Effect of Human Hemoglobin†

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Received June 13, 1984

ABSTRACT: The pH dependence of oxygen affinity of hemoglobin (Bohr effect) is due to ligand-linked pK shifts of ionizable groups. Attempt to identify these groups has produced controversial data and interpretations. In a further attempt to clarify the situation, we noticed that hemoglobin alkylated in its liganded form lost the Bohr effect while hemoglobin alkylated in its unliganded form showed the presence of a practically unmodified Bohr effect. In spite of this difference, analyses of the extent of alkylation of the two compounds failed to identify the presence of specific preferential alkylations. In particular, the α1 valines and β146 histidines appeared to be alkylated to the same extent in the two proteins. Focusing our attention on the effect of the anions on the functional properties of hemoglobin, we measured the Bohr effect of untreated hemoglobin in buffers made with HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], MES [2-(*N*-morpholino)ethanesulfonic acid], and MOPS [3-(*N*-morpholino)propanesulfonic acid], which being zwitterions do not need addition of chlorides or other anions for reaching the desired pH. The shape acquired by the Bohr effect curves, either as pH dependence of oxygen affinity or as pH dependence of protons exchanged with the solution, was irreconcilable with that of the Bohr effect curves in usual buffers. This indicated the relevance of solvent components in determining the functional properties of hemoglobin. A new thermodynamic model is proposed for the Bohr effect that includes the interaction of hemoglobin with solvent components. The classic proton Bohr effect is a special case of the new theory.

One of the most popular equations developed by Wyman (1964) as part of his theory on linked function is the one that correlates the oxygen affinity of hemoglobin with the number of protons exchanged by hemoglobin with the solvent upon ligand binding (ΔH^+):

$$\Delta H^+ = -\frac{\mathrm{d} \log P_{1/2}}{\mathrm{d\ pH}} \qquad (1)$$

This equation is a restatement of the principle of energy conservation. It was shown to be a thermodynamic truism by the quantitative measurements of Antonini et al. (1963). To explain the mechanism of the Bohr effect, German & Wyman (1937) proposed the phenomenological equation

$$\Delta H^+ = \sum_i \left(\frac{K_i}{[\mathrm{H}] + K_i} - \frac{K'_i}{[\mathrm{H}] + K'_i} \right) \qquad (2)$$

which assumes the existence of i specific oxygen-linked groups whose ionization constants change from K to K' upon removal of ligands from hemoglobin solutions.

The shift was attributed to conformational changes of the molecule, producing an intramolecular modification of the electrostatic environment of the groups. Since then, a considerable effort has been made in several laboratories to identify the so-called Bohr effect groups of hemoglobin.

The elucidation of the tridimensional structure of crystalline hemoglobin allowed an approach to the problem by monitoring differences in the structure of liganded and unliganded hemoglobin. These changes were evaluated for their possible electrostatic effects and related to the functional properties of hemoglobins specifically modified in the appropriate residues.

A very good account of this approach has been given by Perutz et al. (1980), and there is very little to add to the reasoning and conclusions there expressed. In partial disagreement with these findings, Ohe & Kajita (1980) using deuterium exchange measured pK shifts of specific groups in liganded and unliganded hemoglobin and calculated that the protons exchanged by those groups largely exceeded the experimental Bohr effect.

Russu & Ho (1980) using NMR spectroscopy reported that the pK of His β146 in (carbonmonoxy)hemoglobin is 8.08 when the protein was in Tris[1] buffers and 7.14 when the protein was in phosphate buffers. Instead, in deoxyhemoglobin the pK of these groups remained near 8.0 in both buffers. Therefore, the histidine at β146 was a Bohr effect group in phosphate and not in Tris buffers. In analogous fashion, results obtained in the laboratory of Gurd (Garner et al., 1963; Matthew et al., 1977; Morrow et al., 1976) showed that Val α1 is a Bohr effect group in solvents containing chlorides, carbonates, and cyanates, while in this laboratory it was demonstrated that it is not so in phosphate buffers (Bucci, 1982).

The relevance of chlorides and other ions to the Bohr effect has been amply documented (DeBruin et al., 1974; Rollema et al., 1975), confirming the determinant role of the anion in solution in the production of the Bohr effect.

These results imply that charged groups in addition to those originally proposed by German & Wyman (1937) and later by Perutz (1970) are involved in the production of the Bohr effect. The participation of a variety of ionizable groups in the Bohr effect is also supported by the computer simulations of Matthews et al. (1979) based exclusively on electrostatic

†Supported in part by Grant HL 13164 of the National Institutes of Health. Computer time and facilities were supported in part by the computer centers of the University of Maryland at College Park and at Baltimore.

[1] Abbreviations: COCM, hemoglobin reacted with bromoacetate as (carbonmonoxy)hemoglobin; DXCM, hemoglobin reacted with bromoacetate as deoxyhemoglobin; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Goode buffers, HEPES, MES, and MOPS.

0006-2960/85/0424-0371$01.50/0

interactions. Blank (1975) proposed a model that explains the Bohr effect of hemoglobin only on the basis of volume changes of the protein, without pK shifts of specific ionizable groups. It appears that the identity of the Bohr effect groups is either controversial or at least elusive to the investigation.

We have made a further attempt to identify, by selective alkylation, the groups responsible for the Bohr effect. We noted that alkylation with bromoacetate of the liganded form of hemoglobin eliminated the Bohr effect, while the latter was only slightly decreased when the chemical treatment was performed in the absence of ligands. The heme–heme interaction was still present in both cases.

This apparently clear-cut situation prompted us to search for the extent of alkylation of the α1 valines and β146 histidines in samples of hemoglobin alkylated either in the liganded or in the deoxy form. Investigations of Perutz et al. (1980), Ohe & Kajita (1980), Gurd (Garner et al., 1963; Morrow et al., 1976; Matthew et al., 1977), and Hill & Davis (1967) indicated that these amino acid residues are Bohr effect groups. In contrast, our analysis indicated that these residues were alkylated to the same extent, about 50%, in both derivatives.

These data, together with the results above mentioned of Russu & Ho (1980) and of Bucci (1982) forced upon us the hypothesis that the Bohr effect is due not to an intramolecular phenomenon affecting specific oxygen-linked ionizations but to the different interaction of oxy- and deoxyhemoglobin with solution components. The proton dependence of the Bohr effect, the mentioned experiments of the Njimegen group (DeBruin et al., 1974; Rollema et al., 1975), and the known effect of polyphosphates and other polyanions on the Bohr effect (Chiancone et al., 1972; Benesch & Benesch, 1967, 1969; Chanutin & Curnish, 1967; Bucci, 1974; Shimizu & Bucci, 1974; Bucci et al., 1978; Salahuddin & Bucci, 1975) focused our attention on the anion components of the solvent. Thus, we measured the Bohr effect in MOPS, MES, and HEPES (Goode buffers), which are zwitterions and do not require titration with acids in order to be adjusted to pH values between 5 and 9.

The Bohr effect in the Goode buffers acquired a very unusual shape totally irreconcilable with the Bohr effect present in Tris buffers adjusted to pH with HCl. A model is proposed that explains the Bohr effect on the basis of the interaction of hemoglobin with ionic effectors present in the solvent.

Materials and Methods

Human hemoglobin was prepared with toluene following the procedure of Drabkin (1946). It was freed of organic and inorganic ions by recycling it through a mixed-bed resin column for 2 h in the cold.

Heme concentration was measured spectrophotometrically on the basis of $\epsilon = 14000\ M^{-1}\ cm^{-1}$ per heme at 540 nm for the CO derivatives of the various heme proteins used. The amount of ferric hemoglobin present in the various mixtures was estimated on the basis of the extinction coefficients reported by Benesch et al. (1965).

Bromoacetate was purchased from Fisher Co. and recrystallized from toluene by addition of petroleum ether. Carboxypeptidase A, treated with diisopropyl fluorophosphate, was purchased from Sigma Chemical Co. All other reagents were analytical grade or better.

Oxygen equilibria were measured spectrophotometrically (Allen et al., 1950) at 20 °C in 0.1 M sodium phosphate buffer at protein concentrations between 1.5 and 2 mg/mL. Alternatively, they were performed with a Hemoscan apparatus at 37 °C with protein concentration near 70 mg/mL. Runs performed with gases at 25 or 35% oxygen gave identical results; therefore, no correction was introduced in the data for dynamic errors.

The number of protons liberated upon oxygenation of deoxyhemoglobin solutions was measured by titration with 0.05 M NaOH until the original pH was restored. These experiments were carried out in 0.25 M NaCl at 20 °C.

Extraction of the terminal valylleucine peptide of the α chains of hemoglobin after partial hydrolysis of the dinitrophenylated protein was accomplished, as described by Rhinesmith et al. (1958). Dinitrophenylation was carried out at pH 7.5 in 0.2 M sodium phosphate or at pH 8.7 in 0.05 M carbonate buffer by incubating the protein with 40–140-fold excess of 1-fluoro-2-dinitrobenzene for 1 h at room temperature. The extracted peptide was hydrolyzed in 6 N HCl for 12 h at 110 °C and the yield of leucine measured with a Beckman 120C amino acid analyzer.

Digestion with carboxypeptidase A was carried out as described by Neer et al. (1968). Analysis of the supernatant demonstrated the presence of only histidine and tyrosine in the ratio 1:1.

Alkylation of hemoglobin was carried out in 0.2 M bromoacetate and 0.66 M sodium phosphate buffer at the desired pH in the presence of 3–5 mg/mL MgO. Under these conditions only the unprotonated histidyl residues are alkylated (Gurd, 1967). The final protein concentration was between 1 and 3%. The necessary amount of deoxyhemoglobin in water was transferred under nitrogen pressure into a container where the alkylating mixture had been deoxygenated by bubbling with nitrogen. The solutions were left at 30 °C for 20 h. At the end of the reaction, carbon monoxide was bubbled in the deoxygenated sample. The solutions were dialyzed in the cold against several changes of 0.1 M sodium phosphate buffer, saturated with CO, at pH 6.7.

Measurements of pH were performed with a Radiometer Model 26 pH meter equipped with a scale expander so that the full-scale pen deflection of a 10-in. Sargent strip chart recorder corresponded to 0.2 units of pH. Spectrophotometric measurements were carried out with a Cary 14 or a Beckman DBG recorder spectrophotometer. A Cary 60 spectropolarimeter was used for circular dichroism measurements. The optical activity of the chemically treated hemoglobins was referred to that of a sample of untreated (carbonmonoxy)-hemoglobin under the same conditions of solvent and protein concentration range. Amino acid analyses were performed with a Beckman 120c automatic analyzer using the standard 4-h run. Protein hydrolysis was done at 110 °C for 22 h in 6 M HCl under vacuum. The extent of alkylation of methionines was measured after treatment of the alkylated proteins with performic acid (Bucci, 1974). Specific carboxyamidomethylation of the β93 cysteines was obtained following the procedure of Guidotti & Konigsberg (1964).

Results

Reaction with Bromoacetate at pH 6.7. We will designate as COCM hemoglobin carboxymethylated in the presence of CO and as DXCM hemoglobin carboxymethylated in the absence of ligands. After carboxymethylation, the samples contained approximately 7 and 13% of ferric form, respectively. Table I shows the number of carboxymethylated residues and the percentage, relative to untreated hemoglobin, of the ellipticity at 222 nm found for COCM and DXCM. The average of three different preparations is reported. The number of histidines modified in positions 1 and 3 in COCM and DXCM appeared quantitatively identical, the number of 1,3-bis(carboxymethyl)histidines, was approximately 1 unit, per $\alpha\beta$ dimer, higher in COCM. No methionyl residue ap-

Table I: Number of Carboxymethylated Residues per $\alpha\beta$ Dimer and Circular Dichroic Activity of Hemoglobin Treated with Bromoacetate

	pH of treatment	% of normal Hb absorbance at 222 nm	1-CM-His	3-CM-His	1,3-bis(CM)-His	CM-Cys	CM-Met	CM-Lys
COCM	6.8	100	1.41	3.14	2.81	0.99	traces	none
DXCM	6.8	100	1.50	3.40	2.02	0.42	traces	none
DXCM	7.6	*a*	1.76	4.75	1.81	0.57	0.92	*b*
DXCM	8.2	95	1.83	4.35	2.70	0.77	0.98	4.5

[a] Not measured. [b] Not calculated.

Table II: Hemoglobin Carboxamidomethylated in β93 Residues[a]

	pH	log *P*	*n*
before treatment with bromoacetate	7.15	0.650	2.90
	8.45	0.040	2.83
after treatment with bromoacetate in the presence of CO	7.15	0.153	2.07
	8.45	0.130	2.07
after treatment with bromoacetate in the absence of ligands	7.15	0.450	1.88
	8.45	0.150	1.82

[a] Hemoglobin was carboxamidomethylated at the β93 residues (19) and subsequently treated with bromoacetate at neutral pH, as described for COCM and DXCM. The Bohr effect and the value of the Hill parameter *n* were measured before and after the reaction with bromoacetate.

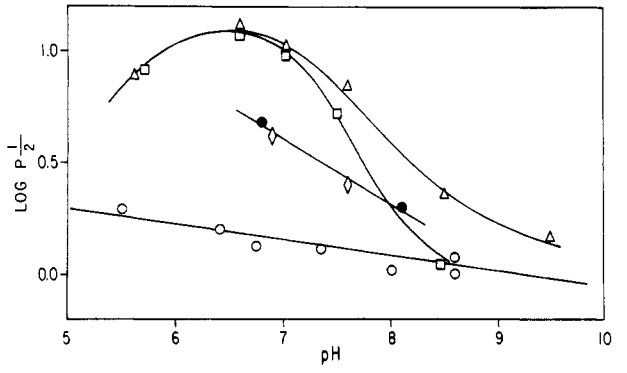

FIGURE 1: Variation of oxygen affinity with pH for (□) control sample of hemoglobin, (△) DXCM obtained at pH 6.8, (◇) DXCM obtained at pH 7.6, (●) DXCM obtained at pH 8.2, and (○) COCM obtained at pH 6.8. Measurements were performed in 0.1 M phosphate or 0.2 M veronal buffer at 20 °C.

peared alkylated. The number of carboxymethylated cysteinyl residues was higher in COCM than in DXCM. For both COCM and DXCM, these were represented only by β93 cysteines. In fact, when the alkylation was performed on hemoglobin previously treated with iodoacetamide as described by Guidotti & Konigsberg (1964) so as to alkylate only these residues, no additional (carboxymethyl)cysteines were produced by the treatment with bromoacetate. Circular dichroism indicated that the chemical treatment did not produce an unfolding of the protein (Table I).

The pH dependence of oxygen affinity of these hemoglobins is illustrated in Figure 1. The value of *n* in the Hill equation was near 2 for COCM and near 1.5 for DXCM. In Figure 1 is also included the Bohr effect of a control sample of hemoglobin treated in the same way as for COCM, except that sodium acetate was substituted for bromoacetate. In this case, *n* was near 2.5. The number of protons liberated at several pH values upon oxygenation of DXCM is shown in Figure 2, where the normal alkaline Bohr effect is also reported.

Table II summarizes the results obtained when oxygen equilibrium curves were measured on samples of hemoglobin that were carboxamidomethylated in the β93 residues (Guidotti & Konigsberg, 1964) prior to the reaction with bromoacetate at neutral pH. The extent of carboxamidomethylation of the β93 residues was proven by amino acid analyses to be

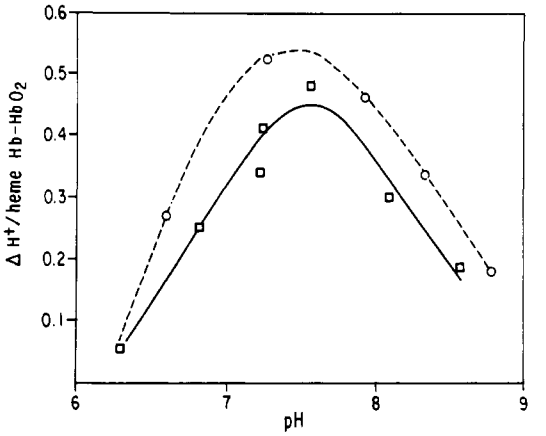

FIGURE 2: Difference in protons bound by the oxy and deoxy derivative of DXCM reacted at pH 6.8 (□). The Bohr effect of normal hemoglobin is also represented (○) (Antonni et al., 1963). Measurements were performed in 0.25 M NaCl at 20 °C.

Table III: Extraction of DNP-valylleucyl Peptide from α Chains of Normal and Carboxymethylated Hemoglobin

protein	excess FDNB per heme	pH of dinitro-phenylation	yield of leucine (%)
normal Hb	120	7.9	70.0
normal Hb	36	8.65	78.6
normal Hb	36	7.5	73.2
DXCM	120	7.9	31.6
DXCM	120	8.65	61.5
COCM	120	7.9	42.6
COCM	120	8.65	57.5

Table IV: Digestion with CPA of Normal and Carboxymethylated Hemoglobin at a Protein to Enzyme Ratio of 60:1

protein	yield of histidine (%)	incubation with enzyme (h)
normal hemoglobin	90.2	3
COCM	36.6	3
COCM	45.9	22
DXCM	46.3	3
DXCM	55.7	22

one per $\alpha\beta$ dimer. Also in this case, the Bohr effect was present only when the subsequent treatment with bromoacetate was performed in the absence of ligands. This indicates that the different functional behavior of COCM and DXCM was not related to the different number of cysteinyl residues alkylated in the two occasions.

The extent of alkylation at neutral pH of the terminal valine of the α chains in COCM and DXCM was estimated by measuring the yield of leucine obtained after extraction of the dinitrophenylated terminal dipeptide according to Rhinesmith et al. (1958). The results are shown in Table III. The low yields obtained with untreated hemoglobin may depend on the partial hydrolysis required by the procedure. The yield decreased in COCM and DXCM, indicating incomplete but extensive alkylation of these residues in the two proteins.

Table IV summarizes the results obtained when COCM and DXCM were digested with carboxypeptidase A. These results

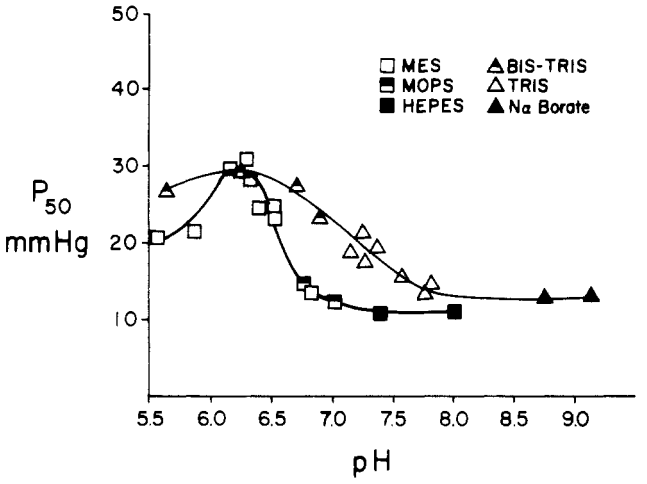

FIGURE 3: Oxygen Bohr effect of human hemoglobin in (▲) 0.15 M Tris-HCl, (top solid triangle) 0.15 M Bis-Tris-HCl, (▲) 0.15 M sodium borate, (top solid box) 0.15 M MOPS, (□) 0.15 M MES, and (■) 0.15 M HEPES. Experiments were at 37 °C, protein concentrations were near 70 mg/mL.

indicate that there was not a significant difference in the extent of alkylation of the β146 histidine in COCM and DXCM. No (carboxymethyl)histidine was present in the supernatant obtained after trichloroacetic acid treatment following digestion with carboxypeptidase, indicating that these derivatives were resistant to the proteolytic activity of the enzyme.

Reaction with Bromoacetate at pH above 7. DXCM was also prepared at pH 7.6 and 8.2. As shown in Table I, the number of alkylated cysteinyl, lysyl, and methionyl residues was higher than that obtained at pH 6.7. The structure of these proteins appeared slightly altered in CD measurement. The Bohr effect, illustrated in Figure 1, although decreased, was not eliminated.

Effect of Organic Anions. In view of the preferential interaction of deoxyhemoglobin with anions and polyanions (Chiancone et al., 1972; Benesch et al., 1967, 1969; Chanutin & Curnish, 1967; Bucci, 1974; Schimuzu & Bucci, 1974; Bucci et al., 1978; Salahuddin & Bucci, 1976), which contribute to the Bohr effect, it was of interest to explore the modification of the Bohr effect produced by zwitterions, in the absence of chlorides or other small fully charged anions. The Bohr effect of hemoglobin A was measured in Goode buffers. In order to cover the necessary pH range, we used MES, MOPS, and HEPES. The interaction of these organic salts with the protein surface should be impaired by the low net charge and by the bulk of their molecules. The results are shown in Figure 3, where the Bohr effect measured in 0.15 M MES, MOPS, and HEPES is compared to that measured in 0.15 M Tris-HCl buffers.

Figure 4 shows the pH dependence of the quantity ΔH^+ as obtained numerically with eq 1 from the data in Figure 3. Clearly, it is impossible to fit the two curves in Figure 4 by using eq 2 with the same values of i, K_i's, and K_i''s.

We are assuming that the interaction of hemoglobin with the Goode buffers was identical, but for the different pH range of their buffering action. A more detailed analysis of the effect of the Goode buffers on the functional properties of hemoglobin is in progress.

Discussion

Carboxymethylation of liganded and unliganded hemoglobin produced two functionally different compounds. The difference was the absence or presence, respectively, of the Bohr effect in the two proteins. In both cases, the heme–heme

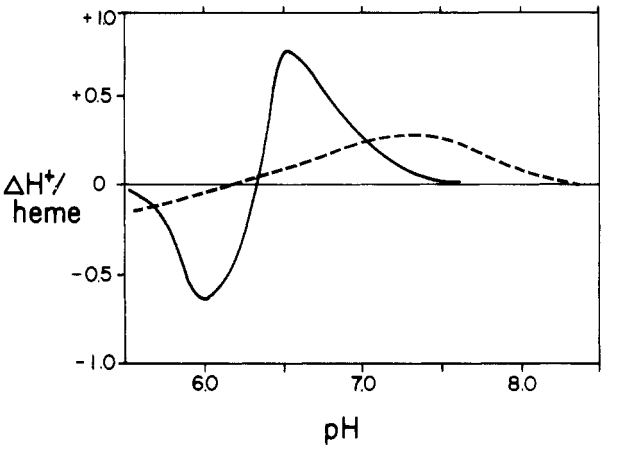

FIGURE 4: Proton Bohr effect computed from the curves in Figure 3 by using the linkage equation, eq 1: (--) 0.15 M Tris, Bis-Tris, and borate; (—) 0.15 M MOPS, MES, and HEPES.

interaction gave values of the parameter n in the Hill plots of at least 1.5. Due to the heterogeneity of the partially alkylated proteins, the detectable Bohr effect and ligand binding cooperativity were minimum effects.

The α1 valines and β146 histidines were incompletely alkylated, approximately 50%, in both proteins, suggesting that the Bohr effect in DXCM was independent from the alkylation of these residues. The β93 cysteines were completely alkylated only in COCM. However, this alkylation per se did not abolish the Bohr effect, as shown by Taylor et al. (1966) and confirmed here.

In regard to histidyl residues, approximately 70% of these residues were alkylated in both COCM and DXCM. This per se cannot justify the absence and presence of the Bohr effect in the two proteins, respectively. However, there was an extra disubstituted histidine per dimer in COCM.

It should be stressed that under the alkylating conditions, 30 °C at ionic strength near 1.5, the pK shift of the Bohr effect groups becomes small (Antonini et al., 1963) and insufficient to produce an all or none alkylation of these residues in either COCM or DXCM, respectively. The considerations are strengthened by the persistence of the Bohr effect in DXCM also when alkylated at pH 8.2, where positive groups are even less protonated.

It may be argued that the histidines responsible for the Bohr effect were not alkylated in deoxyhemoglobin because they were buried in the protein matrix and not exposed to the solvent. In fact, the high ionic strength and the presence of magnesium may have produced a dissociation of only liganded hemoglobin into $\alpha_1\beta_1$ dimer during the alkylation reaction. In this way, some of the residues hidden in the $\alpha_1\beta_2$ interface (other than the valine at α1 and histidine at β146) became available to the alkylation, only in COCM.

This may indeed be the reason why in COCM consistently there was an extra disubstituted histidine per αβ dimer. It should be stressed that the pK shift of only one residue per dimer cannot explain the Bohr effect of human hemoglobin. Also, it is improbable that one of the hidden histidines, found by Steinhardt & Hiremath (1967) to have a pK below 5.0, could be responsible for the alkaline Bohr effect of hemoglobin. Thus, the residue cannot be a pK-shifting Bohr effect group as the traditional model requires. Consequently, if its alkylation is critical to the Bohr effect, it is so because it affects the $\alpha_1\beta_2$ subunit interface and, thereby, the conformation of the system. A possible consequence, as discussed below, would be the interaction of hemoglobin with anions. Admittedly, it is an ad hoc hypothesis.

We believe that attempts to purify pure molecular species from the heterogeneity of COCM and DXCM would have been very costly and useless. In fact, the presence of multiple substitutions was certainly producing isomeric species difficult, if possible, to resolve. Also, in order to understand the functional significance of the various alkylations, we should have purified all of the species for making a statistical evaluation. These statistics are what we present here and is what we were interested to learn.

Effect of Goode Buffers. The gross evidence shown in Figures 3 and 4 is that the functional properties of human hemoglobin can be modulated almost at will by the characteristics of the salts present in solution. The data clearly indicate that it is necessary to develop a new thermodynamic model of general applicability that would include the contribution of solvent components in determining the functional properties of hemoglobin.

A New Model for the Bohr Effect. The molecular mechanism of the Bohr effect as originally proposed by German & Wyman (1973) is expressed by eq 2. They assumed the existence of specific Bohr effect groups, whose pK shift upon ligand binding is an internal affair of the hemoglobin molecule.

The cited results of various authors and those here reported suggest that the parameters i, K_i, and K'_i in eq 2 vary according to the composition of the solvent. A model of more general applicability becomes a necessity.

As noted by Bucci (1976) when ionic effectors capable of preferential interaction with either oxy- or deoxyhemoglobin are present in the solution, they produce a Bohr effect (namely, exchange of protons with the solvent upon ligand binding) that at constant pH obeys the equation

$$\Delta H^+ = \sum_i T_{\max,i}(C'_i - C_i) \qquad (3)$$

where T_{max} is the amount of protons exchanged with the solvent upon saturation of deoxyhemoglobin with each mole of effector (it is supposed that deoxyhemoglobin shows the preferential binding), C and C' are the number of moles of effector bound per mole of protein in oxy- and deoxyhemoglobin, respectively, and i refers to the ith species of effector in the solvent. This can be called the anion Bohr effect.

It can be shown that

$$T_{\max,i} = -\frac{\mathrm{d}\log E_i}{\mathrm{d\ pH}} \qquad (4)$$

where E_i is the free effector concentration at half-saturation of the site i; also

$$C'_i - C_i = \frac{\mathrm{d}\log P_{1/2}}{\mathrm{d}\log E_i} \qquad (5)$$

Substituting eq 4 and 5 in eq 3 we obtain

$$\Delta H^+ = -\frac{\mathrm{d}\log P_{1/2}}{\mathrm{d\ pH}} \qquad (6)$$

which demonstrates the thermodynamic equivalence of eq 1 and 3.

Equation 3 anticipates that the anion Bohr effect depends on the concentration of effectors in solution. In fact, at low effector concentrations, if deoxyhemoglobin is preferred, increasing the effector concentration produces an increase of only the quantity C', while C remains practically at zero. This increases the quantity $(C' - C)$ and the Bohr effect. At high effector concentrations the phenomenon is reversed because deoxyhemoglobin is completely saturated so that C' remains constant, while oxyhemoglobin continues to bind and only the quantity C increases, closing the gap $(C' - C)$, thereby decreasing the Bohr effect.

This phenomenon is evident in the results obtained by Amiconi et al. (1981) using NaCl, by Benesch & Benesch (1967, 1969) using 2,3-diphosphoglycerate, and in the laboratory of Bucci using benzenepentacarboxylate (Bucci 1976; Shimizu & Bucci, 1979; Salahuddin & Bucci, 1976). Bucci (1974) has also measured the quantitative validity of eq 3.

Equation 3 shows that the Bohr effect can be explained solely on the basis of the different interaction of oxy- and deoxyhemoglobin with solvent components. However, the new model does not exclude the proton per se as an effector of hemoglobin, due to the presence of specific Bohr effect groups, independent of solvent components. In that case, in eq 3 $T_{\max,i} = 1.0$, and $C = 1 - K/([\mathrm{H}] + K)$ so that

$$\sum_i (C'_i - C_i) = \Delta H^+ = \sum_i \left(\frac{K_i}{[\mathrm{H}] + K_i} - \frac{K'_i}{[\mathrm{H}] + K'_i} \right) \qquad (7)$$

as in eq 2.

When polyanions interact with hemoglobin, their contribution to the Bohr effect is recognizable and measurable (Bucci, 1974). Otherwise, when monovalent anions like chlorides interact with hemoglobin, the distribution of the Bohr effect between solvent-dependent and solvent-independent groups is difficult to assess, due to the low affinity of hemoglobin for these anions (Chiancone et al., 1972; Fronticelli et al., 1984). Fronticelli et al. (1984) using eq 3, and reasonable values for the number of sites and binding constants of chlorides to deoxyhemoglobin, were able to simulate very well the alkaline Bohr effect of human and bovine hemoglobins. A relevant role of the anions in regulating the Bohr effect would easily explain the different Bohr effects detectable in different buffers.

The equations here elaborated apply only when there is no cooperative binding of either anions or of protons. In this case, the additional parameters to be added to eq 3 would certainly add to the flexibility of the equation in simulating many different experimental findings. Indeed, the sharpness of the Bohr effect present in the Goode buffers suggests the presence of cooperative phenomena in those solutions. The linkage equations developed by Wyman (1964) and tested quantitatively by Antonini et al. (1963) show that, at sufficiently high ionic strength, the energy of the Bohr effect depends exclusively on the net number of protons exchanged by the system with the solvent. Also, the free energy of pK shifts is independent of the nature of the groups involved and the mechanism that produces them. Thus, models based on pK shifts that simulate the spectrum of protons exchanged with pH by hemoglobin upon ligand binding (Chu et al., 1984; Johnson et al., 1984) will correctly describe the energy changes associated with the phenomenon.

It should be stressed that the new theory proposed here does not negate any of the very elegant and onerous experimentations of a number of investigators searching for the Bohr effect groups. It only shows that apparently controversial data may reflect the same truth.

Registry No. MOPS, 1132-61-2; HEPES, 7365-45-9; MES, 4432-31-9; O_2, 7782-44-7.

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Time-Resolved Fluorescence Anisotropies of Diphenylhexatriene and Perylene in Solvents and Lipid Bilayers Obtained from Multifrequency Phase-Modulation Fluorometry†

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Received May 16, 1984

ABSTRACT: Time-resolved decays of fluorescence anisotropy were obtained from frequency-domain measurements of the phase angle difference between the parallel and perpendicular components of the polarized emission and the ratio of the modulated amplitudes. These data were measured at modulation frequencies ranging from 1 to 200 MHz. To demonstrate the general applicability of this method, we describe the resolution of both simple and complex decays of anisotropy. In particular, we resolved single, double, and triple exponential decays of anisotropy and the hindered rotational motions of fluorophores within lipid bilayers. The ease and rapidity with which these results were obtained indicate that frequency-domain measurements are both practical and reliable for the determination of complex decays of anisotropy.

Time-resolved decays of fluorescence anisotropy reveal the time-dependent rotational motions of fluorophores on the nanosecond time scale. These motions are generally dependent upon the fluorophore and its surrounding environment. For instance, time-resolved anisotropies can reveal the torsional motions of tryptophan and tyrosine residues in proteins (Ichiye & Karplus, 1983; Levy & Szabo, 1982; Lipari & Szabo 1980; Munro et al., 1979; Ross et al., 1981a,b), which in turn may reflect the extent of structural fluctuations within the protein matrix. Additionally, time-resolved anisotropies have provided

† Supported by National Science Foundation Grants PCM 80-41320, 81-06910, and 82-10878 to J.R.L. and PCM 79-18646 to E.G.
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0006-2960/85/0424-0376$01.50/0