Bohr Effect in Hemoglobin Deoxy/Cyanomet Intermediates†

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ABSTRACT: The Bohr protons released by oxygen exposure of the unliganded subunits of intermediates $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$ and $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$ were obtained by titrations of concentrated solutions of these species. The Bohr protons released by oxygen exposure of the other intermediates were obtained from titrations of equilibrium mixtures of two parental species, $(\alpha\beta)(\alpha\beta)$, $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$, $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$, and $(\alpha^{+CN}\beta^{+CN})(\alpha^{+CN}\beta^{+CN})$, in which the concentration of the hybrid intermediate was determined by cryogenic electrophoretic techniques. The Bohr effect of the intermediates was calculated by subtracting the Bohr protons released by oxygen exposure of the intermediates from the total Bohr protons of deoxyhemoglobin at the same pH. The Bohr effects of intermediates $(\alpha^{+CN}\beta)(\alpha\beta)$ and $(\alpha\beta^{+CN})(\alpha\beta)$ were similar and vanished at pH 8 where the total Bohr effect of deoxyhemoglobin is still significant. This suggests that the Bohr effect in these intermediates is tertiary in the quaternary T structure. The curve of the Bohr effect of intermediate $(\alpha^{+CN}\beta^{+CN})(\alpha\beta)$, which was close to the curve obtained by adding the Bohr effects of the two monoliganded intermediates at acidic and physiological pH values, was significantly different from the curve obtained by adding the Bohr effects of one liganded subunit of intermediate $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$ and one liganded subunit of intermediate $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$. The Bohr effect of intermediate $(\alpha^{+CN}\beta)(\alpha\beta^{+CN})$ was not determined, but the Bohr protons released by oxygen exposure of the equilibrium mixture of this intermediate and the parental species $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$ and $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$ suggest independent contributions to the Bohr effect of intermediate $(\alpha^{+CN}\beta)(\alpha\beta^{+CN})$ from the Bohr effects of one liganded subunit of each parental species. These findings focus on the functional and structural asymmetry of the diliganded intermediates $(\alpha^{+CN}\beta^{+CN})(\alpha\beta)$ and $(\alpha^{+CN}\beta)(\alpha\beta^{+CN})$, which is predicted by the energetics of the same species [Smith, F. R., & Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82,* 5347–5351; Perrella, M., et al. (1990) *Biophys. Chem. 35,* 97–103; Daugherty, M. A., et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 1110–1114]. The triply-liganded intermediates retained a significant Bohr effect up to physiological pH. The curve of the Bohr effect of intermediate $(\alpha^{+CN}\beta^{+CN})(\alpha^{+CN}\beta)$ was different from the curve calculated by adding the Bohr effects of intermediate $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$ and one liganded β subunit of intermediate $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$. Similarly the curve of the Bohr effect of intermediate $(\alpha^{+CN}\beta^{CN})(\alpha\beta^{+CN})$ was different from the curve calculated by adding the Bohr effects of intermediate $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$ and one liganded α subunit of intermediate $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$. This suggests that the tertiary structures of the liganded subunits in intermediates $(\alpha^{+CN}\beta)(\alpha^{+CN}\beta)$ and $(\alpha\beta^{+CN})(\alpha\beta^{+CN})$ and the triply-liganded intermediates are different, despite the energetics, which indicates that all these species are in the quaternary R structure.

The binding of a heme ligand to hemoglobin induces changes in the tertiary structure of the liganded subunit (Perutz, 1970). The mechanisms by which such changes promote the cooperative binding of the successive molecules of the ligand and finally bring about the quaternary structural change have not yet been fully clarified. Despite recent progress (Ackers et al., 1992; Perrella et al., 1993), the role of subunit tertiary structural changes in the cooperative ligand binding to hemoglobin remains an intriguing functional aspect of this protein. Progress in this area of protein research will provide the tools for a more comprehensive understanding of all cooperative phenomena.

Tertiary and quaternary structural changes in the course of ligand binding to hemoglobin bring about changes in the ionization constants of different groups of the α and β subunits, resulting in the release of protons, or the Bohr effect (Ho & Russu, 1987). The measurement of protons released by the Bohr effect in hemoglobin intermediates is a global probe of such conformational events. In this regard, it is more informative than other more localized conformational probes, such as the reactivity of the thiol groups of the β93 cysteines (Antonini & Brunori, 1971).

We titrated the protons released by exposing to oxygen the hemoglobin intermediates shown in Figure 1, making use of the ferric subunit bound to cyanide as a model of ligation. From the experimental data, we calculated the Bohr effect of the intermediates as the portion of the total Bohr protons lost on changing the state of the subunit from the deoxy to the cyanomet state. The 2-fold purpose of our work was (a) to verify the functional asymmetry of the diliganded molecules previously observed in the energetic studies of the deoxy/cyanomet system (Smith & Ackers, 1985; Perrella et al., 1990a; Daugherty et al., 1991) and (b) to relate the tertiary structural changes of the intermediates, monitored by the Bohr protons, to the quaternary T and R structures of the intermediates, predicted by the energetics.

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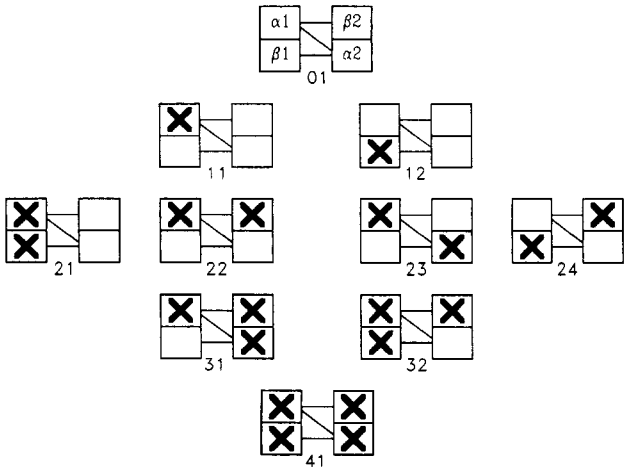

FIGURE 1: Topology of the 10 ligation states in tetrameric hemoglobin. The subunits in the cyanomet form of ligation are denoted by X. The configuration of the α and β subunits with respect to the intersubunit contacts is shown in species [01]. Note: the topology of species [31] and [32] is inverted with respect to that adopted by Ackers and colleagues [see Doyle and Ackers (1992)].

EXPERIMENTAL APPROACH

The study of the hemoglobin intermediates is difficult because of the dissociation and association reactions of the heme ligands, which make all intermediates unstable, and the dissociation of tetramers into dimers. Intermediates that dissociate into dimers in different states of ligation, also called asymmetric or hybrid species, disproportionate into a mixture containing the parental or symmetrical species. Conversely, two symmetrical species can exchange dimers, yielding a mixture containing the hybrid species. Ligand rearrangement and dimer exchange reactions can be slowed at temperatures below zero, but under physiological conditions, they are faster than the rates of all conventional methods of protein separation. The problem of ligand rearrangement can be circumvented by using cyanide bound to the ferric heme of an oxidized subunit as a form of ligation. Since asymmetrical cyanomet intermediates disproportionate into their symmetrical parental species under physiological conditions, it is only possible to study the physicochemical properties of these intermediates in a mixture containing the two parental species. If the composition of the mixture is known, the properties of the hybrid can be obtained by subtracting the contributions of the parental species, which can be studied separately in a pure form. Cryogenic separation techniques allow quantitative analyses of such mixtures (Perrella & Rossi-Bernardi, 1981; Perrella et al., 1981).

Our strategy was to titrate the protons released on oxygen exposure of anaerobic aqueous solutions of the purified parental species [01], [23], and [24] (Figure 1) and of 1 to 1 mixtures of these species and species [41], yielding at equilibrium the hybrid species [11], [12], [21], [22], [31], and [32]. To determine the compositions of the mixtures, hemoglobin A_0 was replaced by hemoglobin S as one of the two parental species and the mixtures were injected into a cryosolvent at −30 °C to quench the dimer exchange reactions before cryofocusing. Hemoglobin S has the same dimer exchange properties as normal hemoglobin (Pettigrew et al., 1982), but the mutation of residue 6β Glu to 6β Val on the surface of the protein allows the separation by cryofocusing of the two parent hemoglobins and their hybrid, having one normal and one mutated dimer, into three well-resolved components, which can be quantitated. Knowing the compositions of the equilibrium mixtures, we determined the contribution of each component to the amount of protons released by oxygen exposure of the mixture.

Clearly, the error in the calculated amount of protons released by oxygen exposure of the asymmetrical intermediate was greater than the error in the amount of protons released by the pure parental species. However, the measurements were accurate, and the error was carefully estimated.

MATERIALS AND METHODS

Preparation of the Purified Parental Species. Hemoglobins A_0 and S were purified by ion exchange chromatography from human red blood cell lysates as previously described (Perrella et al., 1992). Hb^+ [1] was prepared by oxidation of HbO_2 with 1.2 equiv of FeCy at pH 6.8 and purified from products of incomplete oxidation by ion exchange chromatography, as described below. CN^-Hb^+ was prepared by equilibration of Hb^+ with 0.2 M KCl, 1 mM KCN. Species $[23]_{ox}$ and $[24]_{ox}$ in oxy form were prepared in batches by partially oxidizing 4.5 g of HbO_2 with 0.5 equiv of FeCy for 5 min at 4 °C at pH 6.8. All successive procedures were carried out at 4 °C. The partially-oxidized solution was gel filtered on Sephadex G-25 in 5 mM potassium phosphate containing 0.5 mM NaEDTA, pH 6.8, and placed on a column (8 × 40 cm) of CM52 cellulose (Whatman, Ltd., England) equilibrated in the same buffer. Elution was carried out for 16–20 h with 7.5 mM phosphate buffer containing 0.5 mM NaEDTA, pH 7.5, at 300 mL/h. HbO_2 was eluted in a few hours, while Hb^+ was absorbed on the top of the resin bed. The two well-resolved partially-oxidized species were recovered from the resin extruded from the column via a batch procedure. The resin was suspended in 20 mM TRIS-HCl buffer, 50 mM KCl, 1 mM KCN, pH 7.5, to transform the partially-oxidized species into the more stable cyanomet intermediates. A one-column chromatography would normally yield about 0.75 g of species [23] and 0.5 g of species [24]. The two species were equilibrated in 0.2 M KCl, 1 mM KCN and concentrated to 8 mM (heme concentration). Aliquots were stored in liquid nitrogen until use.

Controls of the Purity of the Preparations. Cryofocusing of species $[23]_{ox}$ and $[24]_{ox}$ in oxy form, prior to the addition of cyanide, indicated that these species were contaminated by small amounts, <5% of the total, of other species (Samaja et al., 1987). Controls of the reproducibility of the preparations and their stability upon storage in liquid nitrogen were carried out as follows. Parent species and mixtures of parent species and their respective hybrid were routinely checked spectrophotometrically by measuring the percent of chains in the cyanomet and CO forms immediately preceeding the titrations. Aliquots of hemoglobin were added to CO-saturated 50 mM phosphate buffer containing 1 mM KCN, pH 7.5, in a Thurnberg cuvette, and the absorbance was measured at 540 and 568 nm. In Table 1 are reported the values obtained for the various preparations. Alternatively, the ratio of the extinction coefficients of parental species [23] and [24] in oxy form at two wavelengths, e.g., 576 and 542 nm, was compared with the value calculated from the extinction coefficients of pure HbO_2 and CN^-Hb^+. This method was adopted in other laboratories, where the same derivatives were

[1] Abbreviations: Hb^+, methemoglobin; CN^-Hb^+, cyanomethemoglobin; $N_3^-Hb^+$, azide methemoglobin; Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carboxyhemoglobin; FeCy, potassium ferricyanide; [*ij*], intermediate in which the liganded subunit is in the cyanomet state; $[ij]_{ox}$, oxidized subunits of the intermediate are not bound to cyanide; B.e., Bohr effect.

Table 1: Percent Composition of CO and Cyanomet Chains in the CO-Exposed Hemoglobin Preparations[a]

species	chains	
	% CN^-met	% CO
[01]	1.5 ± 0.5	98.5 ± 0.5
[23]	58.0 ± 1.5	42.0 ± 1.5
[24]	47.5 ± 1.0	52.5 ± 1.0
[01] + [23] + [11]	30.0 ● 1.0 (25)	70.0 ± 1.0 (75)
[01] + [24] + [12]	27.0 ± 1.0 (25)	73.0 ± 1.0 (75)
[01] + [41] + [21]	53.0 ± 1.5 (50)	47.0 ± 1.5 (50)
[23] + [24] + [22]	52.0 ± 0.8 (50)	48.0 ± 0.8 (50)
[23] + [41] + [31]	80.0 ± 1.2 (75)	20.0 ± 1.2 (25)
[24] + [41] + [32]	74.0 ± 1.4 (75)	26.0 ± 1.4 (25)

[a] In parentheses are the expected values in the mixtures of parental species and hybrid.

Table 2: Purity of Species [23] and [24] in Oxy Form: Comparison of Preparations by Ion Exchange Chromatography and Chain Recombination

			experimental	
		calcd	stock solution	before titration
$\alpha_2^{+CN^-}\beta_2^{O_2}$	$\epsilon_{576}/\epsilon_{542}$	0.86	0.846 (0.846)[a]	0.844
	$\epsilon_{577}/\epsilon_{540}$	0.87	0.842 (0.86)[b]	0.838
	% α^{+CN^-}	50	57.2 (53)[b]	58.0
$\alpha_2^{O_2}\beta_2^{+CN^-}$	$\epsilon_{576}/\epsilon_{542}$	0.86	0.880 (0.89)[a]	0.875
	$\epsilon_{577}/\epsilon_{540}$	0.87	0.904 (0.89)[b]	0.895
	% β^{+CN^-}	50	46.7 (46)[b]	47.5

[a] M. A. Shea and G. K. Ackers, personal communication. [b] Miura and Ho (1982).

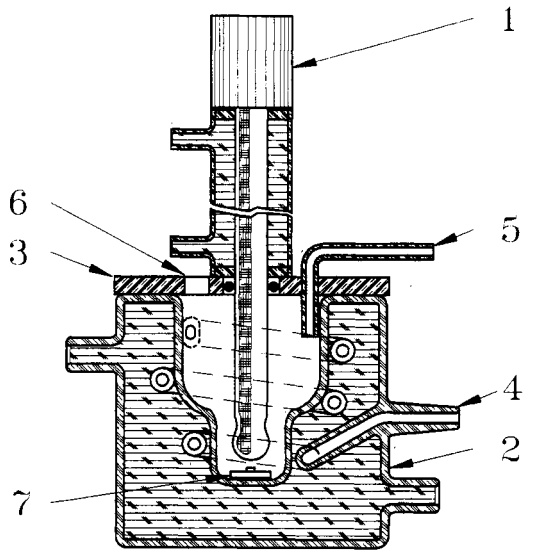

FIGURE 2: Titration vessel. 1, Water-jacketed combined calomel–glass electrode; 2, water-jacketed vessel; 3, vessel cover; 4, nitrogen inlet; 5, oxygen inlet; 6, gas outlet; and 7, magnetic bar.

prepared by combining stoichiometric amounts of isolated subunits in oxy and cyanomet form. The results are listed in Table 2.

Titration of Bohr Protons. A schematic figure of the pH measuring vessel is shown in Figure 2. The vessel and the pH electrode (combined calomel electrode GK 2321C, Radiometer, Copenhagen) were thermostatted by circulating water at 20 °C. Anaerobicity was maintained by a flow of humidified nitrogen at the same temperature. The vessel content was stirred by a magnetic bar. The electrode was calibrated with standard buffers. To titrate, the vessel was filled with 1.00 mL of a deoxygenated solution of hemoglobin. The pH value was recorded, and the nitrogen flow was replaced by an oxygen flow. A stable value of pH was reached within 2 min, and the released protons were titrated with CO_2-free 20 mM NaOH using a 1 μL graduated syringe (CR700-200, Hamilton Co., Reno). One microliter of 20 mM NaOH corresponded to about 0.001 pH unit under the conditions of our experiments, and the stability of the pH measurements was ±0.001 pH unit. Deoxygenation of hemoglobin solutions was carried out in Radiometer glass tonometers. Handling of anaerobic solutions and measurement of sample volumes were carried out by gas-tight Hamilton syringes.

Equilibration of Mixtures of Parental Species. The pH of paired mixtures of the parental species [01], [23], [24], and [41] (Figure 1) was adjusted to the appropriate value by careful addition of 10 mM HCl or 20 mM NaOH in 0.2 M KCl to aliquots of stirred HbO_2 solutions at 0 °C. Deoxygenation of the mixtures was carried out in a tonometer thermostatted at 20 °C. Parental species that hybridized in <1 h, such as mixtures of species [41] and [23] or [24], yielding species [31] or [32], respectively, were equilibrated in the tonometer. Equilibrations requiring up to 70 h were carried out in glass vials with an inlet and outlet for nitrogen flow, fitted with a 5 mm thick silicon rubber cap. The vials, five to seven, were kept in a water bath at 20 °C and interconnected by rubber tubings to a nitrogen cylinder ensuring a continuous flow of humidified gas at 20 °C. One vial routinely contained hemoglobin A_o to serve as a control of the reproducibility of the measurements. Although solutions contained 1 mM CN^-, controls were carried out by adding an excess of CN^-, up to 10 mM, to make sure that the oxidized subunits were fully saturated with the ligand at all pH values.

Cryogenic Focusing. The anaerobic equilibrium mixtures of hemoglobins A_o and S and their hybrid were injected into a stirred cryosolvent, 20 mM TRIS-HCl buffer, pH 7.5, diluted with ethylene glycol to 50% (v/v), saturated with O_2, and cooled at −30 °C to quench the dimer exchange reactions (Perrella & Rossi-Bernardi, 1981). The three components were then separated by cryofocusing at −25 °C on gel tubes for 15–20 h (Perrella et al., 1981). The gel slices containing the components were eluted in 20 mM NaOH, 10 mM KCl, and 50% (v/v) pyridine, and the heme was assayed (Perrella et al., 1993). After a sample of the anaerobic mixture was quenched, the remaining mixture was exposed to oxygen to let the mixture reach rapidly a new equilibrium at which the three components should be present in statistical proportions. A sample of this solution was also analyzed by cryofocusing and served as a control of the stability of the hybrid in focusing. The proportion of hybrid in aerobic equilibrium with the parental species was found to be in the range of 48–50% of the total as predicted by the statistical distribution. Slight differences in the proportion of hybrid with respect to the statistical distribution were used to correct the molar fraction of hybrid observed in the anaerobic experiments.

Calculation of the Protons Released by Oxygen Saturation of the Unliganded Subunits of the Asymmetric Species in the Mixtures. To calculate the protons released by oxygen saturation of species [11], [12], [21], [31], and [32], the experimental data on the protons released by the parental symmetrical species [01], [23], and [24] and the mixtures of these parental species and their respective hybrid were fitted by polynomials of degree 4. The experimental values of the molar fractions of hybrid, f_{Hyb}, and parental species, f_{Par1} and f_{Par2}, as determined by cryofocusing, were fitted similarly by polynomials. The value of the protons/tetramer released by

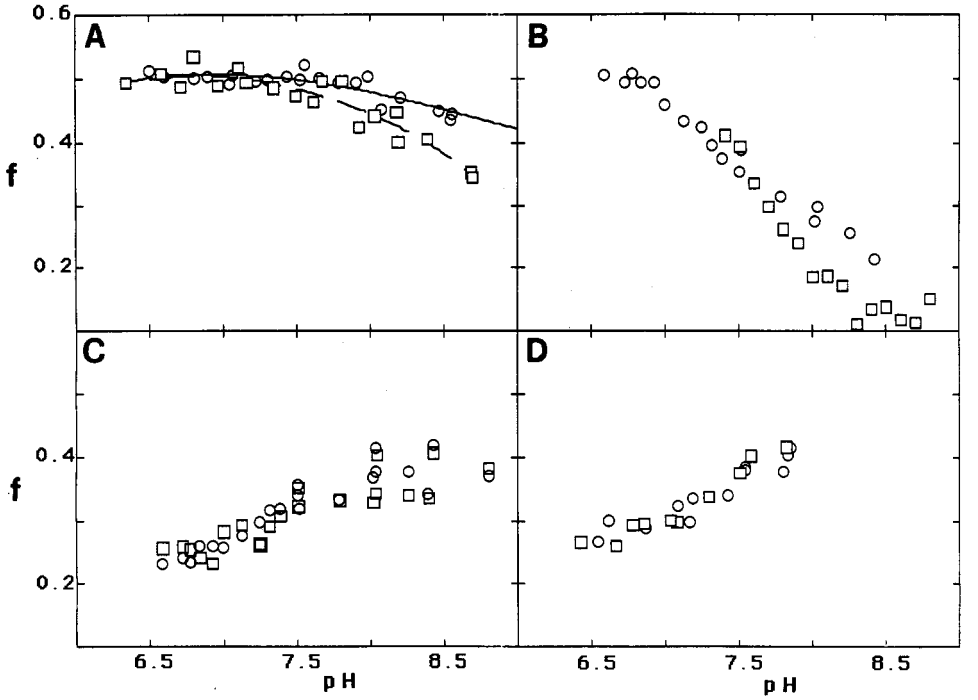

FIGURE 3: Molar fractions, f, of species in anaerobic mixtures vs pH. A, Species [11] (□) in equilibrium with [01] and [23] and species [12] (○) in equilibrium with [01] and [24], the full lines are fitted polynomials; B, species [21] (○) in equilibrium with [01] and [41], (□) from Daugherty et al. (1991); C, species [01] (○) and [41] (□) in equilibrium with [21]; D, species [31] (○) in equilibrium with [23] and [41] and species [32] (□) in equilibrium with [24] and [41].

the hybrid, H^+_{Hyb}, at any experimental value of pH was calculated by the equation

$$H^+_{Hyb} = (H^+_{Mix} - H^+_{Par1} f_{Par1} - H^+_{Par2} f_{Par2})(1/f_{Hyb}) \quad (1)$$

where H^+_{Mix}, H^+_{Par1}, and H^+_{Par2} are the protons/tetramer released by the mixture of parental species and their respective hybrid and singly by the two parental species and f_{Hyb}, f_{Par1}, and f_{Par2} are the molar fractions at equilibrium of the three species, calculated at the same pH value using the polynomials.

The errors, σ, in the released protons and molar fractions of species calculated from the polynomials were obtained from the equation

$$\sigma = [(1/(N-n-1))\sum_{i=1}^{N}(Y_{exp_i} - Y_{pol_i})^2]^{1/2} \quad (2)$$

where N is the number of experimental data, n is the degree of the polynomial, and $Y_{exp_i} - Y_{pol_i}$ are the differences between the experimental and calculated values of protons or molar fractions of species at the same pH value. Such errors were used to determine the errors of the calculated protons released by the asymmetrical intermediates using standard formulas for error propagation.

RESULTS

Equilibrium Mixtures of Parental Species and Hybrid. The fractions of species formed at equilibrium in 1 to 1 mixtures of parental species vs pH, as determined by cryofocusing, are shown in Figure 3. The data on the fractions of hybrids [11] and [12] in panel A were fitted by two different polynomials. The data on hybrid [21], shown in panel B, were in close agreement with previous data obtained under similar conditions (Daugherty et al., 1991). Panel C shows the fractions of parental species [01] and [41] in equilibrium with hybrid species [21]. Data on the fractions of both parental species were pooled and fitted using the same polynomial. The data on the fractions of hybrids [31] and [32] in panel D were also pooled and fitted with one polynomial.

Protons Released by Oxygen Saturation of Hb and the Unliganded Subunits of the Intermediates. Figure 4A shows part of the 100 data points collected on the Bohr protons released by deoxyhemoglobins A_o and S. The average error calculated by fitting a polynomial of degree 4 through the data was ±0.064 protons/tetramer. The curve of the Bohr protons of Hb calculated from titration data previously obtained under similar conditions (Antonini et al., 1965) is shown in Figure 4A for comparison. Experimental data were obtained on the protons released upon oxygen exposure of species [23] and [24] and mixtures of the parental species and their hybrid, equilibrated under anaerobic conditions. The protons released by oxygen exposure of intermediates [11], [12], [21], [31], and [32] were calculated from these data and the data on the fractions of hybrid and parental species using eq 1, as described in Materials and Methods, and are shown in Figures 4B–D and 5. Part of the data (30–50%) were obtained using hemoglobin S as one of the parental species. These data were the same, within the error, as the titration data obtained using hemoglobin A_o. The same solutions containing hemoglobin S were used for the cryogenic experiments.

The protons released by hybrid [22] were not calculated because of the large amount of hemoglobin S needed to produce either parent species [23] or [24] in the mutant form, as required for the cryogenic experiments.

Bohr Effect of the Intermediates. We define the Bohr effect of the intermediates, B.e., as the protons/tetramer lost by the subunits that undergo the change from the deoxy to the cyanomet state. The B.e. of any intermediate was obtained by subtracting the protons released by the intermediate exposed to oxygen, Figures 4B–D and 5, from the total Bohr effect of hemoglobin at the same pH value (Figure 4A). Such calculations were carried out by the use of polynomials fitting

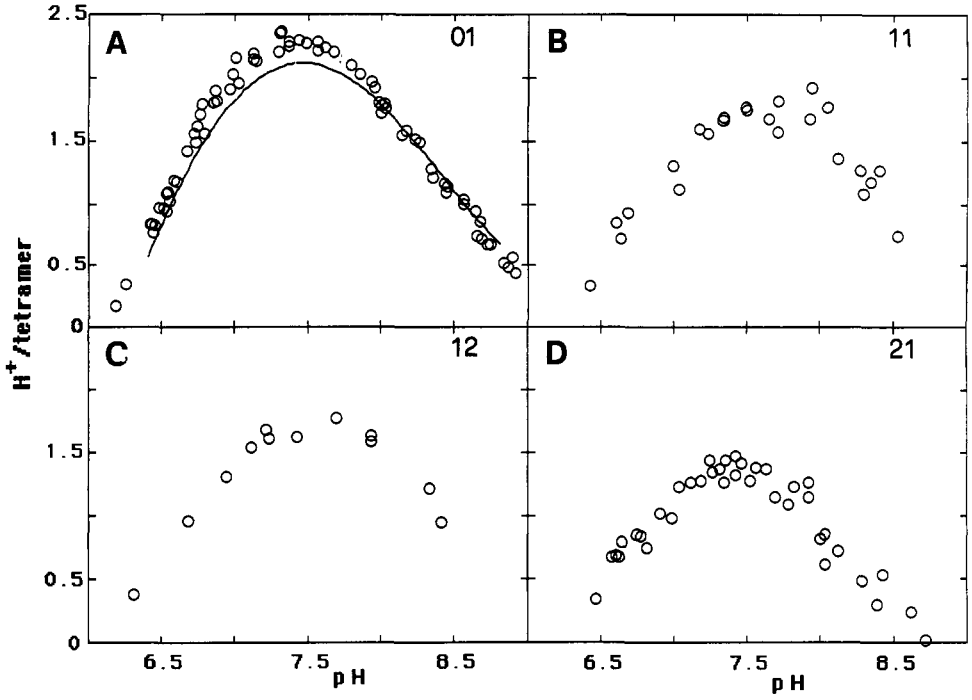

FIGURE 4: Protons/tetramer released on oxygen exposure of unliganded subunits of the species indicated in the upper right corner of each panel. Conditions: 0.20 M KCl, 20 °C, 10 g/dL protein concentration. A, Curve calculated from the data of Antonini et al. (1965), 0.25 M NaCl at 20 °C and 0.5–1 g/dL protein concentration.

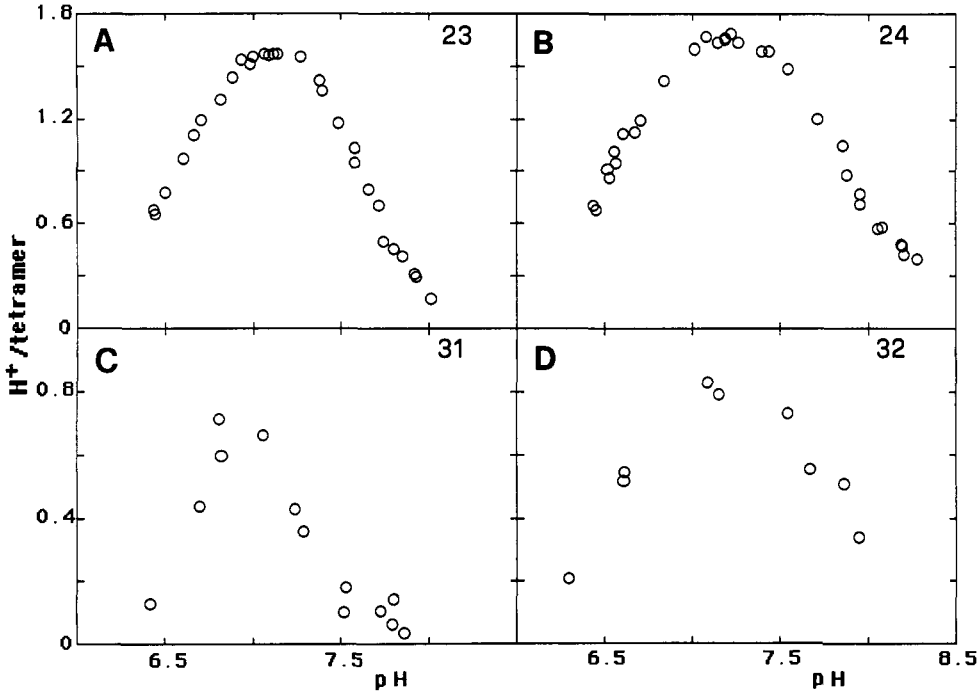

FIGURE 5: Protons/tetramer released on oxygen exposure of unliganded subunits of the species indicated in the upper right corner of each panel. Conditions are as in Figure 4.

the data in Figures 4 and 5. The curves of the B.e. of the various intermediate species are shown in Figure 6 as broad shaded curves, the width of which accounts for the calculated error (see Materials and Methods). Also plotted in Figure 6A,B is the total Bohr effect of hemoglobin. Obviously, the difference between the total Bohr effect and the B.e. of any intermediate at the same pH value yields the protons released by the unliganded subunits of the intermediates reported in Figures 4 and 5.

Figure 7 shows the protons released by oxygen exposure of 1 to 1 mixtures of species [01] and [41] in equilibrium with species [21] and 1 to 1 mixtures of species [23] and [24] in equilibrium with hybrid [22] compared with the sum of the theoretical values of protons released under the same conditions by one β subunit of species [23] and one α subunit of species [24].

Figure 8 summarizes the protons/tetramer lost for each step of ligation at pH 7.4 along all the possible pathways from Hb to CN^-Hb^+. To calculate the protons in the pathways involving species [22], it was assumed that the B.e. of this species was equal to half of the sum of the B.e. of species [23] and [24]. A justification is provided in the discussion below.

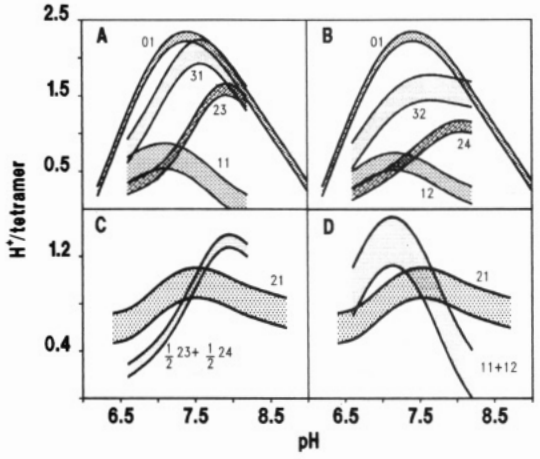

FIGURE 6: Bohr effect, B.e., of intermediates (see text) vs pH. A, Total Bohr effect of Hb (also shown in panel B) and B.e. of species [11], [23], and [31]; B, B.e. of species [12], [24], and [32]; C, B.e. of species [21] and calculated half-sum of B.e. of species [23] and [24]; D, B.e. of species [21], same as C, and calculated sum of B.e. of species [11] and [12]. Shaded areas indicate the error.

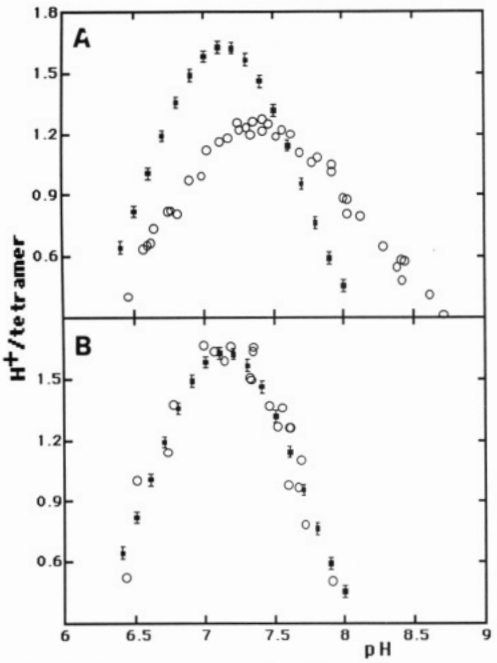

FIGURE 7: Protons/tetramer released on oxygen exposure of unliganded subunits in 1 to 1 mixtures of parental species in equilibrium with hybrid. A, Mixture of species [01], [21], and [41] (O) (the molar fractions of each species are shown in Figure 3B,C); B, mixture of species [22]–[24] (O). In both panels are shown the calculated protons/tetramer released on oxygen exposure of the unliganded subunits of a nonequilibrium 1 to 1 mixture of species [23] and [24] (■). The bars indicate the calculated error.

DISCUSSION

Rationale for the Determination of the Bohr Effect of the Intermediates. The rationale for the use of cyanide bound to the ferric subunit to mimic gaseous ligands, such as O_2 or CO, is based on the crystallographic studies of Hb^+, $N_3^-Hb^+$, and HbCO, which indicate that these proteins have the same quaternary R structure (Perutz & Mathews, 1966; Perutz et al., 1978a,b; Heidner et al., 1976). In these studies, slightly different tertiary structures were also observed due to the different spin state of the iron, which is low in crystals of $N_3^-Hb^+$ and HbCO and high in crystals of aquomethemoglobin. In solution, the spin state of the iron of Hb^+ changes from high to low with increasing pH and is related to changes

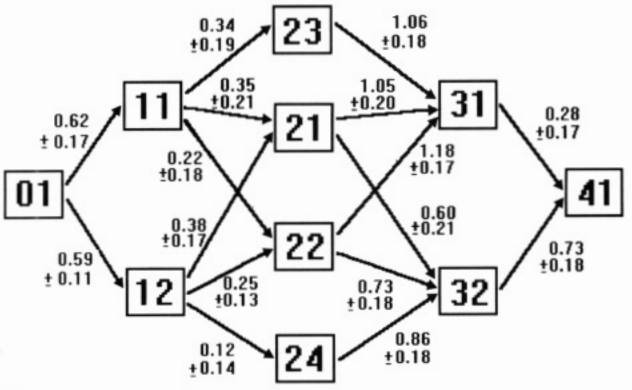

FIGURE 8: Protons/tetramer lost for each step of ligation at pH 7.4 along the various pathways from Hb to CN^-Hb^+. The total Bohr effect under these conditions was 2.30 ± 0.064.

occurring in the tertiary structures of the subunits (Perutz, 1972). Cyanomethemoglobin should have the same quaternary R structure of HbCO, HbO_2, Hb^+, and $N_3^-Hb^+$ and has the low-spin state of the iron of HbCO, HbO_2, and $N_3^-Hb^+$.

Furthermore, deoxygenated solutions of CN^-Hb^+ exposed to oxygen do not release protons, and our titrations were carried out in the presence of an excess of cyanide to ensure that the oxidized subunits were saturated with cyanide under all conditions.

Since it was not possible to measure the protons released upon changing the subunit from the deoxy to the cyanomet state, we used the remaining deoxy subunits as indicators of the Bohr protons in the deoxy/cyanomet system. Thus we defined the B.e. of the intermediates as protons lost on changing the state of the subunit from the deoxy to the cyanomet state, in the sense that these protons are the portion of the total Bohr effect of hemoglobin not accounted for when the subunits are in the cyanomet state.

Clearly the cyanomet state, as any other form of ligation, is not identical to oxygen binding. Although it is known that the quaternary structures of HbO_2 and CN^-Hb^+ are similar, information about the tertiary structural changes induced on ligation by different ligands at intermediate stages of ligation is not available. Thus our data should be extrapolated to predict the Bohr effect in the hemoglobin–oxygen intermediates, keeping in mind that although the rules of the mechanisms of hemoglobin combination with the various ligands may be qualitatively similar, the quantitative translation of these mechanisms into function is different.

Bohr Effect of Monoliganded Intermediates. Figure 6A,B shows that the change from the deoxy to the cyanomet state of one α or one β subunit in intermediates [11] or [12], respectively, brought about a loss of 25–40% of the total Bohr effect, reaching a maximum around the physiological pH. At pH 8.2, the B.e. of these intermediates was hardly significant within the error of the measurements, while the total Bohr effect was more than 60% of the Bohr effect at physiological pH. The Bohr effects of the two intermediates could not be distinguished quantitatively because of the magnitude of the experimental error. The B.e. at the first stage of ligation at pH 7.4, 0.62 ± 0.17 mol of H^+/tetramer from the α subunit and 0.59 ± 0.11 from the β subunit, Figure 8, is remarkably close to the value 0.64 ± 0.07 reported for oxygen ligation under similar conditions (Chu et al., 1984) and agrees qualitatively with the model-dependent values reported by Lee et al. (1988).

Bohr Effect of Diliganded Intermediates. The B.e. of intermediate [23], shown in Figure 6A, and the B.e. of

intermediate [24], shown in Figure 6B, indicate that at pH values <7.5, the change from the deoxy to the cyanomet state of two α or two β subunits resulted in the loss of approximately as many Bohr protons as observed in species [11] and [12]. At pH ≥8, the B.e. of intermediate [23] was the same as the total Bohr effect, while the B.e. of intermediate [24] was about 65% of the total Bohr effect.

Figure 6C shows that the B.e. of species [21] accounted for a large fraction of the total Bohr effect (40–50%) up to physiological pH and was equal to the total Bohr effect at pH ≥8.7. The curve of the B.e. of species [21] is not similar to the curve obtained by the summation of the Bohr effects of one α and one β subunit of species [23] and [24], respectively, as indicated in Figure 6C. The same conclusion is reached by considering directly the experimental data. The protons released by oxygen exposure of the unliganded subunits in the equilibrium mixture of species [01], [21], and [41] did not match the protons that would be released under the same conditions by a nonequilibrium 1 to 1 mixture of species [23] and [24], as shown in Figure 7A.

The protons released by oxygen exposure of the unliganded subunits in equilibrium mixtures of species [23] and [24] were in excellent agreement with those calculated for a nonequilibrium mixture of the same species under the same conditions, as shown in Figure 7B. The equilibrium mixture contains species [22] in statistical proportion with species [23] and [24] at pH 7.4 (Perrella et al., 1990a) and most likely at other pH values. Thus Figure 7B strongly suggests that the B.e. of species [22], contrary to species [21], is equivalent to the combined B.e. of one α and one β subunit of species [23] and [24], respectively.

The overall B.e. for the transition from the mono- to the triliganded stage at pH 7.4 was in the range of 0.95–1.4 mol of H^+/tetramer (Figure 8), somewhat less than the value of 1.62 ± 0.27 reported for oxygen under similar conditions by Chu et al. (1984). The discrepancy is due to the significant B.e. of the triliganded species, which was not observed for oxygen.

Bohr Effect of Triliganded Intermediates. The B.e. of the triliganded species was greater than the B.e. of the diliganded species at all pH values, as shown in Figure 6A,B. It approached the full Bohr effect of hemoglobin in the pH region 7.5–8 in species [31] and at about pH 8 in species [32]. Thus the fourth unliganded subunit, either α or β, retained a significant B.e. at acidic and physiological pH values, as shown in Figure 5C,D. This finding agrees qualitatively with the model-dependent analyses of the data on the oxygen-linked Bohr protons by Lee et al. (1988).

Tertiary and Quaternary Bohr Effects. Crystallographic studies indicate that the $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimers can assume only two alternative configurations yielding the T and R quaternary structures of normal human hemoglobin (Baldwin & Chothia, 1979). However, there is no crystallographic evidence for only two alternative tertiary structures of the subunits, one for each quaternary structure. At intermediate stages of ligation, the tertiary structures of the subunits depend on the quaternary structure of the protein and presumably also on the state of ligation of the subunits within the quaternary structure. In addition, if the tertiary structures of the unliganded subunits in a quaternary structure were influenced by the structures of the liganded subunits, cooperative effects might occur that are not related to the global change in quaternary structure of the protein. These are important questions for the cooperativity of hemoglobin and allosterism in general, which our study of the B.e. of the intermediates addresses.

Since the release of Bohr protons is a global probe of tertiary and quaternary structural effects, it is difficult to distinguish the contributions of the two structural effects in the hemoglobin intermediates without independent information on the quaternary structure of the intermediates. At physiological pH, the energetics of dimer assembly into tetramers of intermediates [11], [12], and [21] is different from the energetics of deoxy- and cyanomethemoglobin, while the energetics of the other intermediate species is the same as that of cyanomethemoglobin (Smith & Ackers, 1985; Perrella et al., 1990a). The extension of the energetic studies to a wider range of pH values has indicated that species [11], [12], and [21] are in a quaternary T structure in which the tertiary structures of the liganded subunits differ from the tertiary structures of the same subunits in deoxyhemoglobin. These studies have also confirmed that intermediates [22]–[24], [31], and [32] are in the quaternary R structure (Daugherty et al., 1991, 1994).

Our study of the Bohr effects of the deoxy/cyanomet intermediates is consistent with the energetics, but it also reveals complex patterns of interaction among the subunits. The intermediates form four distinct groups with regards to the property of the B.e.: (1) species [11] and [12], (2) species [21], (3) species [22]–[24], and (4) species [31] and [32].

The protons lost by hemoglobin upon changing one α or one β subunit from the deoxy to the cyanomet state were a significant part of the total Bohr protons only up to pH 8–8.5. Thus the ionizable groups releasing Bohr protons were completely titrated within this range of pH. At higher pH values, the monoliganded intermediates retained the full potential of deoxyhemoglobin to release Bohr protons (Figure 6A,B). This indicates that such ionizable groups participate in changes of the tertiary structures of the liganded subunits in the quaternary T structure.

The B.e. of intermediate [21] was different from the Bohr effects of the other diliganded intermediates, as discussed above. Up to physiological pH, the B.e. of this species was close to the sum of the Bohr effects of the monoliganded intermediates. These findings are consistent with the energetic studies, which suggest that quaternary T structure for species [11], [12], and [21] (Daugherty et al., 1991, 1994). However, some deviation from additivity, beyond the range of error of the measurements, was observed. This suggests possible interactions between the α and β subunits within the hemoglobin dimer. Above physiological pH, the Bohr effects of the monoliganded species vanished, while the B.e. of species [21] progressively reached the total Bohr effect of hemoglobin (Figure 6D). This may mean that the quaternary T structure of species [21] at physiological pH gradually collapses at alkaline pH into the quaternary R structure of CN^-Hb^+. However, the free energy of dimer assembly into tetramers of Hb at pH >9 remains 3 kcal/mol lower than the free energy of CN^-Hb^+, even though the total Bohr effect vanishes (Antonini et al., 1965) and the quaternary structure is assumed to be T (Daugherty et al., 1991). Thus it cannot be excluded that the quaternary structure of species [21] remains T at pH values where the B.e. of this species vanishes. In such a case, strong interactions at the level of tertiary structures must also occur between the two dimers of species [21].

The energetics of species [22]–[24] in the physiological to alkaline pH range, where the B.e. of these species equals the total B.e. of hemoglobin (pH 8–8.5), indicates that these species are in the quaternary R structure (Smith & Ackers, 1985; Perrella et al., 1990a; Daugherty et al., 1994). In the acidic

to physiological pH range, the Bohr effects of these intermediates were found to be similar to the Bohr effects of species [11] and [12] and significantly smaller than the B.e. of species [21], despite the evidence from the energetic and B.e. studies indicating a quaternary T structure for species [11], [12], and [21]. This surprising finding suggests that if the Bohr effects of intermediates [22]–[24] are due to tertiary structural changes in the quaternary R structure at all pH values, strong interactions of the tertiary structures of the subunits occur in the quaternary R structure.

The energetic studies indicate that the triply-liganded species [31] and [32] are in the quaternary R structure (Smith & Ackers, 1985; Daugherty et al., 1994). Consistent with the energetics are the values of the B.e. of these species which appear to be tertiary effects in the quaternary R structure. Interestingly, the Bohr effects of the triliganded intermediates cannot be calculated from the Bohr effects of species [23] and [24]. The curve of the B.e. of intermediate [31] is significantly different from the curve calculated by the addition of the Bohr effects of intermediate [23] and one liganded β subunit of intermediate [24]. Similarly the curve of the B.e. of intermediate [32] is significantly different from the curve calculated by the addition of the Bohr effects of intermediate [24] and one liganded α subunit of intermediate [23]. This indicates that if the diliganded species [22]–[24] and the triliganded species [31] and [32] have the quaternary R structure, they differ in the tertiary structures of the liganded subunits. The strong coupling of the tertiary structures of the liganded subunits on the two dimers in species [23] and [24], which could be responsible for the observed decrease in Bohr effects of these species as compared with the B.e. of species [21], appears to be mediated by the unliganded subunits on the dimers. Ligation of one such subunit uncouples the interaction and yields the lack of additivity observed in the triliganded species and commented above. More work is needed to confirm such pathways and to understand the nature of the interactions. However, it is clear that probing the tertiary structural changes brought about by ligation adds a new dimension to the problem of hemoglobin allosteric interactions.

Daugherty et al. (1994) have observed that the protons/tetramer lost as Bohr effect in the deoxy/cyanomet intermediates at pH 7.4 along two representative pathways, [01] → [12] → [21] → [31] → [41] and [01] → [12] → [22] → [31] → [41], conforms to the symmetry rule (Ackers et al., 1992). The rule predicts that a significant fraction of the total Bohr protons is released at a stage of any pathway where a transition from quaternary T to quaternary R structure is brought about by ligation of at least one subunit in each dimeric half-molecule. Thus Daugherty et al. (1994) calculated only a minor proton release in step [12] → [21] of the first pathway, in agreement with the assignment of the structure of species [21] to quaternary T. They calculated a major proton release in step [21] → [31] of the same pathway and in step [12] → [22] of the second pathway because species [31] and [22], respectively, are in quaternary R structure. For these calculations, Daugherty et al. (1994) assumed that the dimer Bohr effect of the deoxy/cyanomet intermediates is the same as the dimer Bohr effect measured for oxygen binding by Chu et al. (1984).

Our direct titration measurements indicate that the protons released at pH 7.4 along the two pathways are approximately the same, Figure 8. The discrepancy between the data of Daugherty et al. (1994) and ours is partly accounted for by the experimental error but is also the consequence of the Bohr effect properties of species [22]–[24] at pH values ≤7.5, which we have discussed. The Bohr protons calculated along the same pathways at pH 8, 0.26–0.63–0.87–0.06 and 0.26–1.11–0.39–0.06 protons/tetramer, respectively, or at higher pH values, conform to the predictions of the symmetry rule.

The B.e. is strongly influenced by chloride binding, particularly at physiological pH (Ho & Russu, 1987). We have used a constant chloride concentration of 0.2 M, assuming it to be sufficient to saturate the chloride binding sites at all pH values. Daugherty et al. (1994) used slightly different conditions, namely 0.1 M TRIS-HCl buffer plus 0.1 M NaCl. Since it has been reported that TRIS influences the activity of chloride (Weber, 1992), we cannot exclude that, in addition to the uncertainty due to both experimental errors and approximations in the calculations, some difference in conditions is at the origin of the partial discrepancy between the data of Daugherty et al. (1994) and ours.

Nature of the Bohr Groups in the Intermediates. We do not attempt to speculate on the chemical nature of the groups involved in the B.e. of the intermediates because of the extreme complexity of the problem. We simply note that the change in the shape of the curves of the B.e. of the intermediates, as revealed by the shifts in the maxima, indicates that the nature of these groups cannot be readily predicted by studies of the crystals of Hb and HbO_2 or by NMR studies of solutions of these species. The B.e. of the α and β subunits, close in value in the monoliganded species, was significantly different in species [23], [31] and [24], [32], respectively, as indicated by Figure 6A,B. It appears that double ligation of the α subunits perturbs the tertiary structure of the unliganded β subunit more than double ligation of the β subunits perturbs the tertiary structure of the unliganded α subunit. This finding is consistent with the pH dependence of the mechanisms of the oxidation reaction of Hb by FeCy that we have previously reported (Perrella et al., 1993).

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