The Bohr Effect of Hemoglobin Intermediates and the Role of Salt Bridges in the Tertiary/Quaternary Transitions*

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Understanding mechanisms in cooperative proteins requires the analysis of the intermediate ligation states. The release of hydrogen ions at the intermediate states of native and chemically modified hemoglobin, known as the Bohr effect, is an indicator of the protein tertiary/quaternary transitions, useful for testing models of cooperativity. The Bohr effects due to ligation of one subunit of a dimer and two subunits across the dimer interface are not additive. The reductions of the Bohr effect due to the chemical modification of a Bohr group of one and two α or β subunits are additive. The Bohr effects of monoligated chemically modified hemoglobins indicate the additivity of the effects of ligation and chemical modification with the possible exception of ligation and chemical modification of the α subunits. These observations suggest that ligation of a subunit brings about a tertiary structural change of hemoglobin in the T quaternary structure, which breaks some salt bridges, releases hydrogen ions, and is signaled across the dimer interface in such a way that ligation of a second subunit in the adjacent dimer promotes the switch from the T to the R quaternary structure. The rupture of the salt bridges per se does not drive the transition.

A vast amount of data on the structure/function of human hemoglobin in solution apparently supports the mechanism of a concerted transition between two quaternary structural states in the course of ligand binding, in agreement with the Monod-Wyman-Changeaux model (1). Due to cooperativity, the end states largely prevail on species in a partial state of ligation under equilibrium conditions, masking the functional properties of the intermediate species. This is demonstrated by the close agreement between the isotherms of CO binding calculated from the experimental distributions of the CO ligation intermediates according to the Monod-Wyman-Changeaux model and the alternative Koshland-Nemethy-Filmer model, which assumes transitions through intermediate structural/functional states (2), as shown by Perrella and Di Cera (3). The functional/structural studies of the intermediates still provide the most critical test for any model of cooperativity. Such studies are difficult because of the high rates of the dissociation and association reactions of the physiological ligand, the complexity of the intermediate ligation states Fig. 1, and the instability of tetrameric hemoglobin. The partially liganded hemoglobin tetramers reversibly dissociate into dimers faster than the rate of resolution of the separation techniques (4), and dimer rearrangement reactions occur under nonequilibrium conditions, as depicted in Fig. 2. In a previous study of the Bohr effect of the intermediate ligation states (5), the problem of the ligand mobility was circumvented by using cyanide bound to the ferric subunits to mimic ligation and a cryogenic technique to determine the proportion of any asymmetrical hybrid species in equilibrium with the respective symmetrical parental species (6). This information was needed to calculate the contribution of each species from the total Bohr effect of a mixture of hybrid and parental species. The study of the pH dependence of the Bohr effects of the mono- and diliganded intermediates revealed the absence of additivity of the effects, an important clue to the mechanism of tertiary/quaternary transitions in ligand binding to hemoglobin. However, the discovery by Shibayama et al. (7) that the cyanomet intermediates undergo valency exchange has made such studies questionable. We have now repeated the measurement of the Bohr effect of the mono- and some diliganded species under conditions of slight or negligible valency exchange, confirming the results of the previous study.

Using the same technical approach we have measured the decrease in Bohr hydrogen ions in hemoglobin derivatives in which either one or both Bohr groups of the α and β subunits of deoxy hemoglobin and of the deoxy/cyanomet intermediates were chemically modified by carboxylation (8) and by the NEM1 reaction of cysteine Fβ93 (9). We found that the functional effects of the single and double chemical modifications were additive, as were the combined effects of ligation and chemical modification, with just one possible exception. These findings help define the role of the salt bridges with regard to the stabilization of the hemoglobin T quaternary structure, which was described by Perutz in his stereochemical mechanism of cooperativity (10).

MATERIALS AND METHODS

Hemoglobin Purification—HbA0 was obtained from normal adult blood and HbS from heterozygous donors. The hemoglobins were purified by ion exchange chromatography on CM-52 cellulose, as previously described (5), equilibrated with 0.2 M KCl, and stored in liquid nitrogen at a concentration of 6 mM in heme.

Preparation of NES Hemoglobin—Samples (4.5 g) of HbO2 were reacted with a 5-fold excess of NEM at 4°C and pH 7.3 for 2 h (11). The reactants were gel-filtered on Sephadex G-25 equilibrated with 5 mM potassium phosphate, 0.5 mM Na2EDTA, pH 6.8, and loaded onto a column (8 × 27 cm) of CM-52-cellulose equilibrated with the same

1 The abbreviations used are: NEM, N-ethylmaleimide; Hb, deoxyhemoglobin; HbO2, oxyhemoglobin; CNHb, cyanomet hemoglobin; HbA0, hemoglobin A0; HbS, hemoglobin S; NES HbA0, hemoglobin A0 modified by NEM; BE, Bohr effect.

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purified by chromatography on a CM-52 column as for the purification of sodium pyrogallate. The same procedure was used for the ligated or chemically modified subunits. They are labeled by supergraphic properties as the carbamoylated hemoglobin prepared by the two symmetrical parental species in addition to the asymmetrical associate into identical dimers that reassociate to yield the original dissociate under physiological conditions. Symmetrical tetramers dissociate into different dimers that reassociate, yielding two symmetrical parent species in addition to the asymmetrical species.

Preparation of Hemoglobin Carbamoylated at the α-Amino Groups of the α or β Subunits.—(α′β′)/[αβ]—Hb samples (4.5 g) were reacted anaerobically at 20°C for 1 h with a 50-fold molar excess of KCNO in the presence of O2 traces in the gas flow, N2 was purged through an alkaline pH 6.5, and the excess inositol hexaphosphate was removed by aerobic tonometry and transferred into thermostatted vials for the anaerobic recombination method (8, 14).

Hemoglobin Incubations.—HbO2 solutions were deoxygenated by N2 buffer. Elution with 7.5 mM potassium phosphate, 0.5 mM Na2EDTA, pH 7.3, at 300 ml/h was continued until a good resolution of NES Hb was achieved. The resin-bound derivative was collected and eluted in a batch procedure with 20 mM Tris-HCl, 50 mM KCl, pH 7.5. Titration was achieved. The resin-bound derivative was collected and eluted in a batch procedure with 20 mM Tris-HCl, 50 mM KCl, pH 7.5. Titration was achieved.

Bohr Effect of Native and Modified Hemoglobin Intermediates

FIG. 1. The 10 ligation states of hemoglobin. The non-dissociating dimers (α,β) and (α,β) are shown in brackets. The states are labeled as [ij], indicating the ligation state and the corresponding degeneracy j. Since the α,β and α,β contacts are different, species 21 is structurally nonequivalent to species 22.

FIG. 2. Dimer rearrangement reactions of tetrameric hemoglobin. The ligated or chemically modified subunits are labeled by superscript X. The dashed lines indicate the α,β, and α,β contacts that dissociate under physiological conditions. Symmetrical tetramers dissociate into identical dimers that reassociate to yield the original form. Asymmetrical tetramers dissociate into different dimers that reassociate, yielding two symmetrical parent species in addition to the asymmetrical species.

Measurement of the BE—Samples (1 ml) of 6 mM Hb were transferred into an anaerobic vessel thermostatted at 20°C for pH measurement. The solution was exposed to N2 and the pH was titrated back to the value of the anaerobic sample using carbonate-free 20 mM NaOH (15). BE of the Singly Modified Hemoglobins.—Hemoglobin species chemically modified at a Bohr group of one α or one β subunit cannot be studied in a pure form. Because of the tetramer dissociation reaction, they disproportionate into the parental species, i.e. unmodified and doubly modified hemoglobin, as shown in Fig. 2. The BE of the singly modified species was measured by the same approach used to study the asymmetrical deoxy/cyanomet analogs of the intermediates (5). Since HbS differs from HbA0 for the surface charge but is otherwise functionally equivalent, a one to one mixture of two parent species, e.g. HbS and NES HbA0, was incubated anaerobically until the equilibrium with the asymmetrical species modified by NEM at just one β subunit was reached. The total BE of the mixture was measured, and the contributions to the BE of the fractions of HbS and NES HbA0, at equilibrium were subtracted from the total. The fractions of the three species at equilibrium were measured using a cryogenic separation method, as follows. A sample of the anaerobic mixture was quenched into a hydroorganic solvent at −30°C to stop the tetramer dissociation reactions, the mixture was resolved by cryofocusing at −25°C, and the three fractions were assayed by the pyridine hemochromogen method (16). The data on the rate of equilibration at 20°C in 0.2 M KCl, pH 7, of an equimolar mixture of HbS and (αβ+CN−)(αβ+CN−) plotted in Fig. 3 indicate that equilibrium was reached after a 20-h incubation.

Valency Exchange Controls.—Valency exchange was measured by a procedure similar to that used by Shibayama et al. (7). At the end of each anaerobic incubation of mixtures of deoxy-HbS and cyanomet HbA0 (α±CN−β(α+CN−)β or (α+CN−)β(α+CN−)) valency exchange was stopped by exposure to O2, and the two hemoglobin species, oxy/cyanomet HbS and oxy/cyanomet HbA0, were separated by ion exchange chromatography using small CM-52 columns equilibrated with buffer containing cyanide. The proportion of oxy versus cyanomet hemoglobin in each separated fraction, determined by a spectral analysis of the samples in the 450–600-nm range, yielded the amount of valency exchange. The proportions of each component were obtained by fitting the spectra of the mixtures with the spectra of pure HbO2, CNHb, and Hb+ using a Matlab 5.3 program. The error was 1–2% of the total. Similar spectral analyses of the unmodified oxygenated samples before chromatography allowed a check for the absence of free Hb+ due to cyanide evaporation and for any increase in the total CNHb concentration due to Hb oxidation by O2 leaking in during the anaerobic incubations.

BE of the Deoxy/Cyanomet Analogs of the Intermediates—In the oxyhemyoglobin solutions after incubation. Alternatively, to keep the cyanide concentration during long incubations of the solutions constant, N2 was flown over a 5 mM solution of cyanide before reaching the incubation vial. Oxygen scavenging enzymes, catalase and superoxide dismutase, were not used.

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absence of valency exchange, the anaerobic incubation of HbA0 and species (α′\(\text{CN}\)–β) or (α \(\text{CN}\)–β) should yield the monoliganded intermediates 11 or 12, respectively (Fig. 1). As shown in Fig. 3, the incubation for 3 h at neutral pH of a 1 to 1 mixture of HbS and species (α′\(\text{CN}\)–β) or (α \(\text{CN}\)–β) yielded an amount of hybrid comparable with that observed by incubating under the same conditions HbS and (α′β)\(\text{CN}\)–β(5). The proportion of hybrid was approximately that predicted by the kinetics of the Hb tetramer-dimer reactions (4). A higher proportion of hybrid was obtained when the mixture was pre-incubated under aerobic conditions before deoxygenation (1/2 h) and anaerobic incubation (2 h). Under these conditions the valency exchange and BE were measured using the same anaerobic mixtures.

Data Analysis—The concentration of hydrogen ions released per asymmetrical hybrid in mixture with the parental species was calculated as follows.

\[
[H^+][\text{hyb}] = (H^+[\text{par}_1] - (H^+[\text{par}_2] \times f_{\text{par}_2})) - (H^+[\text{par}_1] \times f_{\text{par}_2}) \times f_{\text{hyb}} \tag{1}
\]

where subscripts refer to the hybrid, parental species 1 and 2, and their mixture with the hybrid, and \(f\) is the fraction of the three species determined by the cryogenic technique. The calculations were carried out on the individual data points or using polynomials fitting the data points. Hence the results presented in the form of bands are calculated interpolations of the original data. The amplitude of the band indicates the error. The difference between the total Bohr hydrogen ions released by HbA0 and the residual Bohr hydrogen ions released by the chemically modified hemoglobins represents the hydrogen ions lost because of the chemical modification. Similarly the difference between the total Bohr hydrogen ions released by HbA0 and the residual Bohr hydrogen ions released by the vacant sites of the liganded species represents the putative hydrogen ions released in the change of the subunit state from deoxy to cyanomet. In such a case the difference is defined as the BE of the ligation intermediate (5).

RESULTS AND DISCUSSION

BE of the Deoxy/ Cyanomet-diliganded Intermediates 23 and 24 and Monoliganded Intermediates 11 and 12—The BE of species 23 and 24 measured after 3 h of anaerobic incubation are compared in Fig. 4, a–b, with the values previously obtained immediately after deoxygenation of the oxygenated species (5). Valency exchange controls were not carried out, but the close agreement with the previous data suggests that the exchange had only minor effects during a 3-h incubation.

The data on the BE of the hybrid monoliganded intermediates, compared in Fig. 4, c–d, with the previously published data (5), were calculated from the titration data of the ternary mixtures of parental species, HbS plus species 23 or 24, and hybrid species incubated under anaerobic conditions for 2 h after deoxygenation (data not shown) and the fractional values of the concentration of hybrid in the mixtures, shown in the inset of Fig. 3. Valency exchange controls carried out at the end of each anaerobic incubation showed negligible exchange at neutral or alkaline pH and slight (≤5%) at the most acidic pH values.2 The valency exchange, proportion of hybrid, and BE were measured using the same anaerobic mixtures.

Data Analysis—The concentration of hydrogen ions released per asymmetrical hybrid in mixture with the parental species was calculated as follows.

\[
[H^+][\text{hyb}] = (H^+[\text{par}_1] - (H^+[\text{par}_2] \times f_{\text{par}_2})) - (H^+[\text{par}_1] \times f_{\text{par}_2}) \times f_{\text{hyb}} \tag{1}
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The curves of the BE in Fig. 4, a–b, and Fig. 4, c–d, are the functional responses of different structures. The bell-shaped curve of the hemoglobin alkaline BE yields the hydrogen ions released in the transition from the T to the R structure due to oxygenation. The bell-shaped curves of the monoliganded intermediates (Fig. 4, c–d) are indicative of the functional effect of a tertiary structural change occurring in the T quaternary structure due to ligation of a subunit. In Fig. 4, c–d, the BE of the vacant sites of the intermediates is measured by the difference between the total BE of Hb and the BE of the monoliganded intermediate. It is clear that these vacant sites released hydrogen ions on oxygenation at all pH values except where the alkaline BE of Hb itself vanishes. The sigmoidal shape of the curves of the BE of the diliganded intermediates (Fig. 4, a–b) are not equal to the sum of the bell-shaped curves of the two monoliganded intermediates, indicating a profound interaction between the ligation sites. The vacant sites of the diliganded intermediates did not release hydrogen ions upon oxygenation at pH values at which the BE of Hb is still significant, Fig. 4, a–b. This is the response of a molecule in the R quaternary structure in which all salt bridges are broken, as described by Perutz (10) in his stereochemical mechanism. At neutral and acidic pH values, the two vacant sites in diliganded species 23 and 24 released on oxygenation an amount of hydrogen ions

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2 L. Benazzi, unpublished data.
comparable or even less than the amount released by the three
vacant sites in the monoliganded species. If the quaternary
structures of these diliganded species were in T/R equilibrium,
the molecules in T structure should have an additive BE twice
as large as the BE of the monoliganded species, and the mole-
cules in R structure should have a BE similar to that observed
at alkaline pH. Such a hypothesis is not consistent with the
experimental data. Instead the sigmoidal curve of the diligan-
ded intermediates is consistent with the hypothesis that the
quaternary structures of diliganded species 23 and 24 have
switched to the R conformation at all pH values. The hydrogen
ions released on oxygenation by the unliganded subunits of
these species would then be the functional effects of tertiary
structure changes modulated by pH. It is not known whether
such effects are associated with the R2 structure discovered in
studying the crystals of carboxy hemoglobin crystallized under
low salt (0.1 M Cl\(^-\)) and acidic (pH 5.8) conditions (18).
However, the crystallographic studies indicate the possible exist-
ance of alternative R quaternary structures.

Our interpretation of the correlation between the observed
functional effects of mono- and diligation and the tertiary/
quaternary structures of the protein is consistent with the
interpretation of the energetics of the same species provided by
Ackers et al. (19). The cooperative free energy of ligand
binding, \(\Delta G_C\), can be measured from the difference between the
free energy changes for the dimer-tetramer assembly of liga-
tion intermediate \(ij\) and Hb, assumed as the reference state,
\(\Delta G_C(ij \rightarrow \text{Hb}) = \Delta G_C - \Delta G_{01}\) (20). At neutral pH the \(\Delta G_C\) value
for the first ligation step in the deoxy/cyanomet Hb analog is 50%
of the value for the transition to CNHb (4). A similar value was
calculated from the distributions of the CO ligation intermedi-
ates reported by Perrella et al. (6, 21) under similar conditions.
At alkaline pH the \(\Delta G_C\) values remain intermediate (22). The
observation of an intermediate \(\Delta G_C\) value for the first ligation
step is consistent with a two state concerted model (23) and has
been interpreted as the energy of destabilization of the T quater-
nary structure due to the binding of one ligand (19). In
contrast, the \(\Delta G_C\) values for the symmetrical diliganded inter-
mediates are the same as for the transition to CNHb in the
range from neutral to alkaline pH. This indicates that these
species are in the R quaternary structure under these condi-
tions and that \(\Delta G_C\) is not significantly modulated by the effects
of pH on the tertiary structure of the unliganded subunits.
Such effects were observed in our functional studies since the
curve of BE versus pH was sigmoidal in shape (Fig. 4). Sigmoid-
al curves of the BE were also observed in the study of the
triply liganded intermediates (5). The present study is partly
consistent with the symmetry rule model for hemoglobin cooper-
ativity proposed by Ackers et al. (19). Important features of
this model are the energetic and other functional properties of
the diliganded intermediate 21 (Fig. 1), which have been re-
cently confirmed to be different from those of the diliganded
species 22, 23, and 24 (24). As discussed above, the low rate of
formation of intermediate 21 from the parental species Hb and
CNHb together with the high rate of valency exchange have
precluded our study of the BE of this key intermediate.

BE of (\(\alpha^C\beta\))(\(\alpha^C\beta\)) and NES HbA\(_{\alpha}\)—The experimental values
of the hydrogen ions released on oxygenation per tetramer of
HbA\(_{\alpha}\) and its doubly modified derivatives at 20 °C in 0.2 M KCl
in the pH range of the alkaline BE are shown in Fig. 5, a–c.

The NEM reaction of Cys393 causes a modification of the
tertiary structure of the \(\beta\) subunit that disrupts a network of
salt bridges at the \(\alpha_1\beta_2\) interface with the participation of His146, as observed in the Hb crystal structure (9, 10). Car-
bamoylation of Val14 breaks a chloride ion-mediated salt
bridge within the structure of the \(\alpha\) subunit (25, 26). The data
in Fig. 5, a–c, indicate that the chemical modification reduced
significantly the BE in each derivative, in qualitative agree-
ment with the values at physiological pH reported by several
authors (8, 9). The loss of Bohr hydrogen ions observed in NES
HbA\(_{\alpha}\), i.e. the difference between the BE of native and chemi-
cally modified hemoglobin, in a range of pH values is compared
in Fig. 6a with the differential titration curve of His146\(_\beta\),
assuming the values \(pK_{\text{deoxy}} = 8.1\) and \(pK_{\text{oxy}} = 7.2\) (27). The
loss of Bohr hydrogen ions in (\(\alpha^C\beta\))(\(\alpha^C\beta\)) is compared in Fig. 6b
with the differential titration curve of Val14\(_\alpha\), assuming the
values \(pK_{\text{deoxy}} = 8.0\) and \(pK_{\text{oxy}} = 7.25\) (28). Also shown in Fig.
6b is the differential titration curve corrected on the assump-
tion that carbamoylation of Val14 perturbs His122\(_\alpha\) (26). The
differential titration curve of His122\(_\alpha\) required for the correc-
tion was calculated assuming the values \(pK_{\text{deoxy}} = 6.1\) and
\(pK_{\text{oxy}} = 6.6\) (29). The simulations in Fig. 6, a and b, indicate the
strict correlation between the rupture of the salt bridges in-
ferrered from the crystal structures of deoxy and oxy hemoglobin
and the functional effects we have measured in the chemically
modified protein.

BE of the Mixtures of Parental Species, Hbs plus (\(\alpha^C\beta\))(\(\alpha^C\beta\))
and Hbs plus NES HbA\(_{\alpha}\) and Hybrid Species—The experi-
mental titration data are shown in Fig. 7, a–b. The fractions of
BE due to the hybrid species (not shown) were calculated from the
data in Fig. 7, a–b, using the values of the fraction of hybrid
species in the mixture shown in Fig. 7, c–d.

BE Loss in the Doubly and Singly Chemically Modified He-
moglobin—The Bohr hydrogen ions lost in NES HbA\(_{\alpha}\) and
(\(\alpha^C\beta\))(\(\alpha^C\beta\)) (Fig. 6, a–b) are compared in Fig. 8, a–b, with those
lost in the singly modified derivatives calculated from the data
in Fig. 7. Within the experimental error, the effects on function
of both types of chemical modification were additive. The ad-
ditivitity observed in this study of the NEM-modified hemoglo-
bin is consistent with the additivity observed in the study of the
energetics of the same species by Ackers and co-workers (30–32).
The modification by NEM of the Bohr group on 1 \(\beta\) subunit

![Fig. 5. Hydrogen ions released at 20 °C in 0.2 M KCl by the alkaline BE of HbAo (a), NES HbA\(_{\alpha}\) (b), and (\(\alpha^C\beta\))(\(\alpha^C\beta\)) (c).]
results in a 1.4-kcal/mol increase in free energy for the dimer-tetramer assembly, one-half the amount observed with a double modification (31, 32), indicating that the two sites are independent of one another with regard to their effect on function despite the destabilization of the quaternary structure brought about by the chemical modification. A measure of such a destabilization is obtained by comparing the change in free energy from Hb to NES Hb, 2.8 kcal/mol, with the free energy change of 5.8 kcal/mol for the transition from Hb to NES HbO2 (32).

BE of the Chemically Modified Deoxy/Cyanomet-monoliganded Intermediates—Fig. 9, a–d, shows the BE of intermediates \((\alpha\beta^\text{CN}-(\alpha')\beta')\), \((\alpha\beta^\text{CN}-(\alpha')\beta)\alpha\beta\text{NEM}\), \((\alpha^\text{CN}-(\alpha')\beta)\alpha\beta\text{NEM}\), and \((\alpha^\text{CN}-(\alpha')\beta)\alpha\beta\text{NEM}\). The fraction of hybrid and parental species modified by the NEM reaction of one \(\beta\) subunit and by carboxamidyla-
tion of one \(\alpha\) subunit in the same mixtures are shown in c and d, respectively.

Role of the Salt Bridges in the Ternary/Quaternary Transitions—From the above information we can draw the following overall picture of the tertiary and quaternary transitions in the process of hemoglobin ligation. Hydrogen ions are released upon ligation of a subunit because ligation perturbs the tertiary structure of the ligated subunit in such a way as to break some salt bridge and/or hydrogen bond in agreement with the Perutz stereochemical mechanism (10). However, ligation is also signaled to the neighboring subunits, since a second ligation step promotes a dramatic change from the T to the R structure, as monitored by the different characteristics and nonadditivity of the BE of the mono- and diliganded interme-

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**Fig. 6. BE of chemically modified HbA.** a, experimental data on the hydrogen ions lost by NES HbA (shaded band) compared with the theoretical curve calculated assuming, for His146b, \(pK_{\text{deoxy}} = 8.1\) and \(pK_{\text{oxy}} = 7.2\) (solid line). b, experimental data on the hydrogen ions lost by \((\alpha^\text{CN}-(\alpha')\beta)\) (shaded band) compared with the theoretical curve calculated assuming, for Val1, \(pK_{\text{deoxy}} = 8.0\) and \(pK_{\text{oxy}} = 7.25\) (solid line) and, in addition to the same \(pK\) values for Val1\(\alpha\), \(pK_{\text{deoxy}} = 6.1\) and \(pK_{\text{oxy}} = 6.6\) for His122\(\alpha\) (29) (broken line).

**Fig. 7.** Hydrogen ions released at 20 °C in 0.2 M KCl by the alkaline BE of all the species at equilibrium in one to one mix-
tures of HbS and NES HbA\(\alpha\) and HbS and \((\alpha\beta)\) (b). The proportions of hy-
brid species modified by the NEM reac-
tion of one \(\beta\) subunit and by carbamoyla-
tion of one \(\alpha\) subunit in the same mixtures are shown in c and d, respectively.

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mediated by a tertiary structural change in the adjacent subunits involving the rupture of a salt bridge. A possible exception was species \((\alpha^\text{CN}-(\alpha')\beta)\) (Fig. 9d). The BE of this species, i.e. the difference between the total BE of hemoglobin and the residual BE of its vacant, normal and chemically modified sites (shaded area in Fig. 9d) was significantly greater than the sum of the BE of the mono-liganded species and the hydrogen ions lost because of the chemical modification of one \(\alpha\) subunit (continuous line in Fig. 9d).
Our observations are also consistent with the observations reported by Bettati and Mozzarelli (33) that silica gel-modified species from the BE of HbA0. The modified species are as follows. a, band I, NES HbA0; band 2, singly NEM-modified HbA0; solid line, one-half value of NES HbA0; band I, (αββSβ)Hb; band 2, (αββSαβ); solid line, one-half value of (αββSαβ).

The rupture of a salt bridge, as it occurs in the chemically modified intermediates from the BE of HbA0. The modified species are as follows. a, band I, NES HbA0; band 2, singly NEM-modified HbA0; solid line, one-half value of NES HbA0.

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FIG. 8. Bohr hydrogen ions lost because of the chemical modifications, as calculated by subtracting the residual BE of the modified species from the BE of HbA0. The modified species are as follows. a, band I, NES HbA0; band 2, singly NEM-modified HbA0; solid line, one-half value of NES HbA0.

FIG. 9. Alkaline BE of the chemically modified monoliganded cyanomet intermediates, as calculated by subtracting the Bohr hydrogen ions of the vacant sites of the chemically modified intermediates from the BE of HbA0 (shaded areas). The experimental data are compared with the theoretical curves calculated by adding to the BE of the monoliganded intermediates (data in Fig. 4, c–d) the Bohr hydrogen ions lost because of the single chemical modification (data in Fig. 8 without error indication). a, experimental data for species (αββSαβ) and calculated curve for species (αββSαβ) plus (αββSαβ); b, experimental data for species (αββSαβ) and calculated curve for species (αββSαβ) plus (αββSαβ). c, experimental data for species (αββSαβ) and calculated curve for species (αββSαβ) plus (αββSαβ). d, experimental data for species (αββSαβ) and calculated curve for species (αββSαβ) plus (αββSαβ).
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14. Russo, R. (1997) Signal transduction in a cooperative protein. Ph.D. thesis, Milan University
15. Benazzi, L., Russo, R., Ripamonti, M., and M., Perrella, M. (1998) Methods Enzymol. 295, 208–227
16. Perrella, M., and Rossi-Bernardi, L. (1994) Methods Enzymol. 232, 445–460
17. Perrella, M., Benazzi, L., Shea, M. A., and Ackers, G. K. (1990) Biophys. Chem. 35, 97–103
18. Silva, M. M., Rogers, P. H., and Arnone, A. (1992) J. Biol. Chem. 267, 17248–17256
19. Ackers, G. K., Doyle, M. L., Myers, D., and Daugherty, M. A. (1992) Science 255, 54–63
20. Ackers, G. K., and Smith, F. R. (1987) Annu. Rev. Biophys. Biophys. Chem. 16, 583–609
21. Perrella, M., Colosimo, A., Benazzi, L., Ripamonti, M., and Rossi-Bernardi, L. (1990) Biophys. Chem. 37, 211–223
22. Daugherty, M. A., Shea, M. A., and Ackers, G. K. (1994) Biochemistry 33, 10345–10357
23. Ackers, G. K., and Johnson, M. L. (1981) J. Mol. Biol. 147, 559–582
24. Ackers, G. K., Holt, J. M., Huang, Y., Grinkova, Y., Klinger, A. L., and Denisov, I. (2000) Proteins Struct. Funct. Genet. 41, Suppl. 4, 23–43
25. Ho, C., and Russu, I. M. (1987) Biochemistry 26, 6299–6305
26. O'Donnel, S., Mandaro, R., Schuster, T. M., and Arnone, A. (1979) J. Biol. Chem. 254, 12204–12208
27. Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., and Ho, C. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1246–1249
28. Van Beek, G. C. M., Zuiderweg, E. R. P., and De Bruin, S. H. (1979) Eur. J. Biochem. 99, 379–383
29. Nishikura, K. (1978) Biochem. J. 173, 651–657
30. Ackers, G. K., and Smith, F. R. (1985) Annu. Rev. Biochem. 54, 597–629
31. LiCata, V. J., Speros, P. C., Rovida, E., and Ackers, G. K. (1990) Biochemistry 29, 9771–9783
32. Turner, G. J., Galacteros, F., Doyle, M. L., Hedlund, B., Pettigrew, D. W., Turner, B. W., Smith, F. R., Moo-Penn, W., Rucknagel, D. L., and Ackers, G. K. (1992) Proteins Struct. Funct. Genet 14, 333–350
33. Bettati, S., and Mozzarelli, A. (1997) J. Biol. Chem. 272, 32050–32055