Comparative Biological Chemistry of Cobalt Hemoglobin*

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SUMMARY

Cobalt hemoglobin A binds oxygen cooperatively with a Hill coefficient of up to 2.3 and its \( p_{1/2} \) is found to be 91 mm. Its oxygen affinity is controlled by diphosphoglycerate which the protein binds very strongly. Cobalt hemoglobin A possesses only 40% of the alkaline Bohr effect of the native enzyme; carbon dioxide has no effect upon oxygen binding. The oxidation-reduction potential of cobalt hemoglobin A is +0.100 volt which is about 50 mV less positive than that of native hemoglobin. The electron paramagnetic resonance of the nitrooxide spin label attached to the sulfhydryl groups of cysteines and the rates of reaction of these groups with paramagnetic species indicate that the "heme" crevice is more open near that residue. A detailed comparison of these properties with those of the native protein suggests subtle and intricate alterations of the quaternary structures of the oxy and deoxy species which result from substitution of the metal atom.

The discovery by Hoffman and Petering (1) that cobalt can be substituted for iron in bovine Hb and sperm whale myoglobin with the retention of reversible oxygen binding property has opened an informative branch of hemoglobin chemistry. The electronic state of the cobalt nucleus in the modified protein can be examined with EPR (1). The single crystal EPR of \( \text{CoMb} \) and \( \text{CoMbO}_2 \) was reported by Chien and Dickinson (2) with the finding that oxygen is \( \pi \)-bonded to cobalt in \( \text{CoMbO}_2 \). In addition, comparison of the properties of the native and the cobaltic proteins should help to elucidate the role of the metal atom in homotropic and heterotropic allosteric interactions.

The initial successful preparation of cooperative \( \text{CoHb} \) was found to be difficult to reproduce. Thus, Hoffman et al. (3) reported that a large percentage of their reconstitutions were noncooperative, with \( n = 1 \), and this problem still remains. Furthermore, both \( \text{CoHb} \) and \( \text{CoHbO}_2 \) are in low spin states (1, 2). If the cobalt protein truly possesses cooperative oxygen binding property, then some revision of the stereochemical theory is indicated. According to the current model stated in the most detailed form by Perutz (4), it is the transition from high spin iron in the deoxy form to low spin iron in the oxy form which triggers the homotropic interaction.

The central objective of this work is to prepare cooperative \( \text{CoHb} \) from human hemoglobin, and to compare the properties of this protein with those of the native species in order to gain further insight into the role of the metal in hemoglobin. In this paper we present the results of oxygen equilibria (with and without DPG), carbon dioxide equilibria, alkaline Bohr effect, ultraviolet-visible spectra, oxidation-reduction potentials of \( \text{CoHb} \), spin label EPR studies, and stopped flow reactions with \( p\text{-MB} \).

EXPERIMENTAL PROCEDURE

Preparation of Human \( \text{CoHb} \)—HbA was prepared from fresh human blood by the method of Perutz (5). \( \text{CoHbA} \) was prepared in 20 ml batches of approximately 1% concentration. This procedure has been described previously (2). Eighty percent of our preparations are cooperative (\( n = 1.8 \) to 2.3). Individual \( \text{CoHbA} \) batches did not seem to depend upon the apooglobin preparation, as both \( n = 1 \) and \( n > 1 \) could result from reconstitution of a given apooglobin preparation. All \( \text{CoHbA} \) batches were routinely analyzed for oxygen reversibility and Hill coefficient. The noncooperative ones were discarded.

\( \text{CoHbA}^+ \) was prepared from cobaltic protoporphyrin IX acetate by essentially the same procedure as above except that sodium dithionite is omitted. Fresh preparations of \( \text{CoHbA}^+ \) tend to leave a trail of free cobaltic porphyrin on a Sephadex column possibly indicating weakness of its binding to the globin. The \( \text{CoHbA}^+ \) preparations are free of \( \text{CoHbA} \) contamination as confirmed by the absence of EPR signals at \( g = 2 \) and 2.3.

Determination of Spectral Properties—The optical spectra of cobalt proteins were obtained with a Cary 14 spectrophotometer. The extinction coefficients given in this paper are calculated on the basis of an extinction coefficient of 7.96 O.D./per cent for deoxy \( \text{CoHbA} \) at 5350 A as determined by lyophilizing several salt free preparations.

Measurement of Oxygen Equilibria—A 1-cm path length cylindrical cell, fitted with stopcocks and containing a small magnetic stirring bar, was used in these measurements. The protein concentration was typically 0.1% in pH 7.2 0.1 M phosphate buffer. Isobesticity at 5640, 5425, and 5270 A was monitored to detect protein denaturation. At 25°C, each sample was used to determine three points on the oxygenation curve. Desired partial pressures of oxygen were achieved by first equilibrating...
the solution with an atmosphere of air or oxygen with constant flushing and stirring for 45 min. The cell was then pumped down to a pressure appropriate to the desired partial pressure of oxygen. Minimum total pressure above the solution was 200 mm and 30 to 40 min with gentle stirring was allowed for equilibration. Complete deoxygenation was accomplished by flushing the stirred protein solution with wet nitrogen gas for 45 to 60 min until no changes were observed in the visible spectrum.

DPG solutions were prepared from the pentacyclohexylammonium salt (Calbiochem, Los Angeles, California, Lot 000444) by treatment with Dowex 50W-X8 (6). Because of the relatively low stability of CoHb in salt free solutions, the DPG binding experiments were performed in 0.1 M NaCl. This salt concentration was selected as a balance between stabilization of the protein and suppression of the DPG effect on PI/Z. Some denaturation occurred as evidenced by the formation of small amounts of precipitate. As a consequence, there was some scatter in the Hill plot. The oxygenation curve was unchanged when our CoHbA was taken through the procedure of Benesch et al. (6) which was devised specifically for the stripping of DPG. Therefore, our preparative procedure effectively removed DPG from the protein.

Determination of Alkaline Bohr Effect—The alkaline Bohr effect was determined by the method of differential titration (7). A thermally jacketed cell was fitted with a pH electrode (Radiometer GK2321) and inlet and outlet for wet nitrogen or oxygen flush. Samples containing 0.33% protein and 1.0 M NaCl were deoxygenated by moderate stirring under nitrogen flush until the pH of the solution had stabilized for 10 min. The pH value was recorded and an oxygen atmosphere introduced. As soon as the drop in pH had stabilized, the solution was titrated back to the initial deoxy value with a 50-µl syringe filled with 0.01 N standardized NaOH.

Measurements were made on CoHbA as well as on native hemoglobin and also on iron reconstituted HbA. The latter was prepared by exactly the same procedure used to obtain cobalt reconstituted HbA.

Measurements of Oxidation-Reduction Potentials—Oxidation reduction potentials were measured potentiometrically in a thermally jacketed cell equipped with 1 cm² platinum electrode and a KCl-bridged saturated calomel electrode. A Honeywell 2730 potentiometer was used to monitor the equilibrium potentials. The procedure is basically the mixture method of Antonini et al. (8).

Solutions of CoHbA and CoHbA⁺ (0.3%) in 0.1 M phosphate buffer were prepared as above. The protein concentration was determined by conversion to the same deoxy form with dithionite followed by spectrophotometry. Equinormal amounts of CoHbA and CoHbA⁺ were mixed under a constant N₂ flush with gentle stirring. Mediator (0.02 mole fraction of cobalt content) was introduced. Of the many mediators tried the ones which gave the most reproducible results and promoted rapid equilibration of potential are methylene blue for pH > 7.3 and thionin for pH < 7.3. Methylene blue can also be used at pH < 7.3. Control runs with native hemoglobin at pH 7.0 reproduced literatures values (9) to within 2 mv. Sodium phosphate buffer (0.1 µ) was used below pH 8; 0.1 M sodium borate buffer was used above pH 8.

Spin Label Studies—Spin labeled CoHbA was prepared by reacting a 1% protein solution in 0.1 µ pH 7.0 phosphate buffer with a 10-fold excess of iodoacetamide-nitroxide spin label (3-(1-iodocacetamide)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, Lot 1201, Syva, Palo Alto, California). After 12 hours at 5°C, excess nitroxide was removed by gel permeation chromatography on a Sephadex G-25 (fine) column (20 cm x 3 cm²) equilibrated with 0.1 M pH 7 phosphate buffer. The EPR spectra of the spin labeled CoHbA were measured on a Varian E-9 spectrometer operating at 9.5 GHz.

Reaction of CoHbA with p-MB—p-Chloromercuribenzoate (Sigma Chemical Co.) was twice reprecipitated with acetic acid, centrifuged, and redisolved in 0.1 N NaOH. The stock solutions are 30 µM p-MB and 2.5 µM hemoglobin tetramer, both solutions being 0.1 M phosphate pH 7.0. The kinetics of PMB reaction with the sulfhydryl groups of hemoglobins was followed by monitoring the change in absorbance at 2540 nm which accompanies the formation of mercaptide. A Gibson-Durrum stopped flow apparatus was used for this work.

RESULTS

Optical Properties of Cobalt Hemoglobins—The optical spectra for CoHbA and CoHbA⁺ are given in Figs. 1 and 2. The spectra for FeHbA and FeHbA⁺ are also included for comparison. The Fe superscript is included in the abbreviations of the native species for the sake of clarity. The visible band of FeHbA is a single broad peak at 5550 Å; the corresponding absorption of CoHbA is slightly lower in intensity and is characterized by a}

![Fig. 1 (left). Optical spectra of FeHbA and CoHbA in pH 7.2 0.1 M phosphate buffer at 25°C. Ten-fold reduction of protein concentration was used to record spectra below 4500 Å. * values apply to spectrum above 4500 Å.](image1)

![Fig. 2 (center). Optical spectra of FeHbA⁺ and CoHbA⁺ in pH 7.2 0.1 M phosphate buffer at 25°C. Ten-fold reduction of protein concentration was used to record spectra below 4500 Å. * values apply to spectrum above 4500 Å.](image2)

![Fig. 3 (right). Optical spectrum of CoHbA⁺ in pH 7.2 0.1 M phosphate buffer at 25°C. Ten-fold reduction of protein concentration was used to record spectrum below 4500 Å. * values apply to spectrum above 4500 Å.](image3)
shoulder at about 5230 Å. The latter feature was not resolved by Hoffmann and Petering (1) for bovine CoHb, recent preparation of horse CoHb was reported to have a shoulder at 5150 Å (10). The extinction coefficients at peak maximum for CoHbA and FeHbA are virtually the same at 13 O.D./millimolar-heme. The α and β bands of CoHbA are found at 5660 and 5350 Å as compared to 5770 and 5300 Å in the case of FeHbA. The extinction coefficient for the α band of CoHbA is 0.8 of that of the visible band of FeHbA; the corresponding ratio is 1.2 for the native hemoglobin. The minimum between the α and β bands of FeHbA is much deeper than that of CoHbA.

The optical spectra of CoHbA+ (Fig. 3) bears a close resemblance to the spectrum of CoHbA0 (Fig. 2). The 6300 Å absorption of met hemoglobin is totally absent in CoHbA+. Furthermore, the α and β bands of CoHbA+ and CoHbA0 are found at nearly identical wave lengths with almost the same extinction coefficients. In fact, if one were unaware of these facts, one could be misled into concluding that CoHbA+ has a very strong affinity for oxygen when trying to deoxygenate a sample contaminated with the oxidized species.

The native protein has apparently little or no affinity for F- and Cl- ions. Introduction of these ions at 0.1 M to CoHbA1 caused no discernible change in its visible spectrum. However, it does have some affinity for the CN- ion. After introducing CN- to CoHbA+ and after long standing, a stable spectrum results (Fig. 4) which is quite similar to the one for methemoglobin cyanide (11).

Cooperative Binding of Oxygen—A typical oxygenation curve for CoHbA is shown in Fig. 5. The Hill coefficient is 2.3; the half-saturation partial pressure of oxygen is 91 mm. Also included in the figure is the curve for FeHbA (12). A Hill plot for CoHbA is given in Fig. 6.

A physiologically important heterotropic linkage phenomenon of hemoglobin is the modification of oxygen affinity by organic phosphates. The effect of DPG on the oxygen affinity of CoHbA was investigated. In order to perform this experiment, the CoHbA protein which was prepared in phosphate buffer must first be thoroughly dialyzed to remove all the phosphate ions. It was found that the resulting salt free protein solution is rather unstable. There was rapid coagulation of CoHbA at room temperature. Therefore, the measurements with DPG were made in 0.1 M NaCl. The pH of the solution was 7.3 before deoxygenation at 25°. As mentioned above our CoHbA is stripped in preparation.

Like FeHbA (6), the affinity of CoHbA for oxygen decreases with increasing DPG concentration (Table I). However, unlike the native protein, the effect for CoHbA is linear with the DPG concentration and saturates at about 1 mole of DPG per mole of tetramer. In the case of FeHbA the effect is not saturated until the DPG concentration is about three times the hemoglobin concentration even in salt free solutions. The results in Table I show that each CoHbA tetramer binds 1 mole of DPG as is the case for FeHbA, and that the CoHbA-DPG complex is more stable than the FeHbA-DPG complex. Identical decrease of oxygen affinity by DPG has been reported for horse CoHb (10). The relative decrease for FeHbA, CoHbA, and horse CoHb by saturation amounts of DPG, expressed as Δ log p50, is 0.42, 0.48 and 0.48, respectively. Since the DPG effect on horse CoHb was not determined at less than saturation amount of DPG (10), it cannot be said whether the horse CoHb-DPG complex is also more stable than the native hemoglobin complex.

Alkaline Bohr Effect—The native hemoglobin releases 2.5 protons per tetramer upon oxygenation at physiological conditions (7). It is this alkaline Bohr effect which promotes the transport of CO2. CoHbA is rather unstable in salt free medium and denaturation could invalidate the measurement. Additional protons could be released when other residues become exposed in the denatured form. Because of this concern, the Bohr effect was studied in 1.0 M NaCl. Of course high salt concentration is known to lower the absolute alkaline Bohr effect. Therefore, parallel measurements were made on native hemoglobin under identical conditions. Such a comparison would give the relative Bohr effect of CoHbA referenced to the native

### TABLE I

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<th>[DPG]</th>
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![Fig. 4 (left). Optical spectrum of CoHbA-CN in pH 7.2 0.1 M phosphate buffer and 0.1 M NaCN at 25°. Ten-fold reduction of protein concentration was used to record spectrum below 4500 Å. ε values apply to spectrum above 4500 Å.

![Fig. 5 (center). Oxygenation curves for FeHbA and CoHbA in pH 7.3 0.1 M phosphate buffer at room temperature. The data for FeHbA are taken from Reference 12, also reproduced in this work.

![Fig. 6 (right). Hill plot of CoHbA data of Fig. 5.](http://www.jbc.org)
FIG. 7 (left). Alkaline Bohr effect of native human hemoglobin A (○), iron reconstituted HbA (△), and CoHbA (○) as determined by differential titration (7). In each case the protein concentration was 0.33/m in unbuffered 1.0 M NaCl.

FIG. 8 (center). Optical spectra of CoHbA in the presence and absence of CO2 in 0.1 M pH 7 phosphate buffer at 25°. The curve enzyme. This information will be more valuable than some uncertain value obtained for the physiological conditions.

The CoHbA solution has a pH of 6.82 which drops to 6.77 when fully oxygenated. A total of 0.6 Bohr protons are released (Fig. 7). Under the same conditions, native human hemoglobin A yields 1.5 Bohr protons. Therefore, cobalt substitution causes 60% reduction of this heterotropic allosteric interaction.

The possibility that the lowering of the Bohr effect may be due to reconstitution procedure producing proteins not exactly in the native conformation was considered. The following experimental findings tend to discount this possibility. Apo-globin was reconstituted with ferrous protoporphyrin IX. Several of these reconstituted FeHbA were found to possess effectively all of the normal Bohr effect (Fig. 7).

A reduction of the Bohr effect should be accompanied by a similar diminution of CO2 affinity. Classically, the carbamino CO2 combined with hemoglobin is determined by barium precipitation. However, this method requires more CoHbA than can be obtained readily. An alternative method of Magaria and Green (13), in which the change in oxygen affinity at constant pH produced by CO2 is measured, was used instead. The change is believed to be due to the formation of oxygen-linked carbamino compounds since the bicarbonate ion itself causes little or no change in oxygen affinity (14, 15). Fig. 8 shows that the introduction of 5% CO2 in a CoHbA02 solution in 0.1 M pH 7.3 phosphate buffer reduces the fraction of CO2Hb by only 5%. For comparison under somewhat different conditions (14), there is a 40% decrease of oxygenated native hemoglobin by the same amount of CO2.

Hoffman et al. (3) have reported that human and bovine CoHb (α ~ 1) have 50% of the native Bohr effect, but horse CoHb has a normal Bohr effect (10).

**Oxidation Reduction Potentials for CoHbA**—The oxidation-reduction potential for the HbA/HbA+ couple of the native enzyme at physiological pH is +0.147 volt (8, 9). Using the designated CO2 was equilibrated to 722 mm of air plus 38 mm of CO2.

**Oxidation-reduction potential of CoHbA (○) and CoHbA (○) as a function of pH. The CoHbA data are from mixture method of Reference 8 and reproduced in part in this work.**

The same conditions employed by Antonini et al. (8), we have measured the oxidation-reduction potentials of CoHbA, CoHbA+ couple as a function of pH. The results are summarized in Fig. 9 along with those for the native enzyme (10). We also checked the latter values at several pH values. The potentials are referred to the normal hydrogen electrode.

The oxidation-reduction potential for CoHbA is +0.100 volt. It should be realized that $E_{1/2}$ is the exact equivalent of $(RT/F) \ln \alpha$ (15). The value of $E_{1/2}$ calculated from the oxygen affinity data is +0.11 volt.

The slopes of the $E_{1/2}$ versus pH for the two proteins are identical at -0.06 volt per pH. To explain a similar dependence in cytochrome c, Theorell and Åkeson (16) suggested that the reduction is accompanied by dissociation of the reduced species. The proposed mechanism, adapted to hemoglobin (15), can be represented by

$$rHb^+ + H^{\alpha} \rightleftharpoons rHb{^\alpha}H \rightleftharpoons rHb + H^+$$

(1)

where $\alpha$ is the degree of dissociation. Then the potential is given as

$$E = E_0 + \frac{RT}{nF} \ln \left[ \frac{[\alpha]}{[red]} \right] + \ln \left[ \frac{1}{K} - pH \right]$$

(2)

where $K$, the equilibrium constant for Equation 1, is $<<[H^+]$ and $E$ is independent of pH when $K \gg [H^+]$. If this interpretation is correct, then both the iron and the cobalt proteins have the same heme-linked acid groups. They differ, however, somewhat in their pK values which are 6.5 for FbHbA and 6.9 for CoHbA. This small difference could have significant consequence, among others, in determining the conditions for crystallization of the two species (17).

**Spin-labeled CoHbA**—The EPR spectrum of iodoacetamide-nitroxide spin labeled CoHbA is shown in Fig. 10a. The spectral
intensity corresponds approximately to two nitroxide radicals per tetramer when compared to solutions of free spin label at known concentrations. The spectrum indicates a single type of binding site for the nitroxide. Oxygenation of the sample results in no discernible changes in the EPR spectrum. That is oxy and deoxy $\text{CoHb}_A$ have identical spin label EPR spectra. From the widths of the $m_t$ lines as compared to those of free nitroxide and no further spectral changes upon additional purification of the spin-labeled $\text{CoHb}_A$, the label can be definitely said to be bound to the protein.

The above observations are to be contrasted with the results of Ogawa (18) for native hemoglobin labeled with the same nitroxide. (Fig. 10, b and c). In this case, the spectra are much more anisotropic and there are dramatic differences between the oxy and deoxy species.

It is apparent from comparison of Fig. 10, a, b, and c that the attached spin labels differ in their mobilities in the two proteins. The EPR spectra of spin-labeled native enzyme showed that the label is immediately immobilized; it is only weakly immobilized in $\text{CoHb}_A$.

Reactions of $p$-MB with $\text{CoHb}_A$ - To further investigate the protein conformation in the vicinity of the heme binding site, the rate of reaction of $p$-MB with the cys(92)$\beta$ -SH groups was measured. The results are summarized in Table II. Our results for $\text{FeHb}_A$ are in excellent agreement with those of Geraci and Snda (19). There are two notable features about the $\text{FeHb}_A$ data. Whereas the second order rate constant for $\text{FeHb}_A$ and $\text{FeHb}_A_0$ are nearly the same, the rates for both cobalt species are even greater than that for $\text{FeHb}_A_0$. The results show that the space in the vicinity of cys(92)$\beta$ in the cobalt protein is more open than it is in the native protein.

![Fig. 10. Electron paramagnetic resonance spectra of spin-labeled (a) $\text{CoHb}_A$ and $\text{CoHb}_A_0$; (b) horse $\text{FeHb}$; (c) horse $\text{FeHb}_A$ at pH 7. b and c are from Reference 18 and also reproduced for $\text{FeHb}_A$ in this work.](image)

**TABLE II**

<table>
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<th>Protein</th>
<th>$k (10^3 \text{M}^{-1} \text{s}^{-1})$</th>
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<tr>
<td>$\text{CoHb}_A$</td>
<td>19 ± 2</td>
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<tr>
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<tr>
<td>$p\text{FeHb}_A$</td>
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**DISCUSSION**

It has been established beyond doubt that properly reconstituted $\text{CoHb}_A$ binds oxygen cooperatively. The Hill coefficient of our best preparation is 2.3. This, when compared with $n \approx 2.8$ for native hemoglobin indicates that cobalt substitution lowers cooperativity by about 18%. It is possible, but not very likely, that cooperativity of $\text{CoHb}_A$ may be raised further with better reconstitution. In any event, the invariant contacts between the $\alpha$ and $\beta$ chains, i.e. $\alpha_\beta$ and $\alpha_2$, are probably unperturbed by the cobalt substitution. Most of other linkages responsible for homotropic allosteric transition are also intact.

The affinity of $\text{CoHb}_A$ for oxygen is about 14 times smaller than $\text{FeHb}_A$ (Fig. 5). Even larger differences in oxygen affinity of about 100-fold have been reported between $\text{CoMb}$ and $\text{FeMb}$ (20). For these proteins, the difference is directly attributable to the fact that the standard free energy change of oxygenation is 2.4 cal mole$^{-1}$ more positive for the cobalt species (20). The reduction of oxygen affinity upon cobalt substitution is unique among modified hemoprotein. All other modifications result in a large increase in oxygen affinities. This includes modifications of the porphyrin, sulphydryl, and amino moieties of the protein (21, 22).

Both $\text{CoHb}_A$ and horse $\text{CoHb}$ (10) retain the full DPG heterotropic linkage properties. The binding of DPG by $\text{CoHb}_A$ appears to be much stronger than $\text{FeHb}_A$. The biochemical data of Bunn and Brichl (23) together with fitting of molecular models (4) suggest that the DPG binding site involves ValVal(1)$\beta$, lysiE6P(82)$\beta$, and hiniH21(143)$\beta$. However, because the affinity of DPG for native hemoglobin is totally inhibited at the salt concentrations used to precipitate crystals of deoxyhemoglobin, x-ray study of its DPG complex is not possible. The affinity of DPG for $\text{CoHb}_A$ is much less sensitive to salt. It may be possible to pinpoint the DPG binding site with x-ray technique on the cobalt protein.

There is a large reduction of alkaline Bohr effect when $\text{CoHb}_A$ is compared with native and iron reconstituted $\text{FeHb}_A$ under identical conditions. There is an accompanying decrease in CO$_2$ affinity with cobalt substitution. The implication is that there are far fewer salt bridges in the deoxycobaltohemoglobin than in the deoxyferrohemoglobin. A supporting evidence is that the pH of unbuffered $\text{CoHb}_A$ in 1.0 M NaCl is 6.82 compared to 7.33 for $\text{FeHb}_A$ under the same conditions.

Among the eight salt bridges postulated to exist in $\text{FeHb}$ (4) the ones between the imidazolium group of hisHC3(146)$\beta$, and aspFG1(94)$\beta$, are most readily subjected to scrutiny. A nitroxide spin label attached to cys(93)$\beta$ provides a most sensitive probe for the environment in its vicinity. Its EPR spectrum reveals that the mobility of the spin label becomes less restrained when the enzyme is oxygenated (18). This supports the idea that the above mentioned salt linkage is disrupted in the oxy state. The EPR spectra of $\text{CoHb}_A$ (Fig 10a) shows that there is little restriction to the local motion of the spin label in this species. The label in this protein has even more mobility than it has when attached to $\text{CoHb}_A$. We take this to mean that there is no His-Asp linkage in the $\beta$ chains of the cobalt species. The fact that the spin label EPR spectrum is unchanged by oxygenation should not be construed as there is no conformational changes in the vicinity of the label. The label has so much free space to move around in the deoxy state that any increase of this space in the oxy state cannot further reduce the EPR line widths.

The reaction rates with $p$-MB are entirely consistent with the
The reaction rates are faster for CoHbA and CoHbO₂ than for FeHbA and FeHbO₂ indicating that the site of reaction is more accessible in the cobalt species. The small difference in the rate constants could mean that the environment in the vicinity of cys(93)β is slightly less restrictive in CoHbO₂ than it is in FeHbA.

The orientations of the heme plane in FeMb and CoMb are the same within the experimental accuracy of EPR (2). The same appears to be true in FeHbA and CoHbA (17). Therefore, it is probable that the COOH-terminal histidine has been moved to a new position by cobalt substitution. This displacement could conceivably sever the αβ salt bridges between the α-carboxyl group of histC3(146)β and the ε-amino group of lysC5-.

A number of salt bridges undisrupted by cobalt substitution. This displacement should be sufficiently small to permit the cobalt atom to fit into the "central hole" of the porphyrin (3, 24) and is probably not trans to recycle the enzyme. It is valid to inquire whether the oxidative stability of the Co(II) complexes is retained in the cobalt protein. Our results show that, if anything, CoHbA is more easily oxidized than FeHbA. The near equality of the oxidation-reduction potential of the two proteins suggest that the porphyrin moiety and globin environment almost completely determine E₁/₂ and that E₁/₂ is an index of oxygen reversibility.

In conclusion, CoHbA shares many of the biological properties of the native enzyme. It is, however, an inferior oxygen carrier with diminished proton and CO₂ linkages. Organic phosphates are less able to control the oxygen affinity of CoHbA than FeHbA because they form stable complex in the former. CoHbA has lower oxygen affinity and oxidative stability than the native species.

Acknowledgments—The technical assistance of Miss Lisa Chien and the assistance of Dr. J. Bemis with the stopped flow experiments are acknowledged.

REFERENCES


After this paper was submitted, a paper by Hsu et al. appeared in which they reached a similar conclusion.

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