Electron Transfer between Hemoglobin and Coboglobin Mediated by Methylene Blue

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Abstract: Electron transfer between ^{Co}Hb and $^{Fe}Hb^+$ and between ^{Fe}Hb and $^{Co}Hb^+$ as mediated by Methylene Blue was followed with EPR. The equilibria involved are: $^{Co}Hb + M\ (k_{-1}) \rightleftharpoons {}^{Co}Hb^+ + M\cdot\ (k_1)$; $^{Fe}Hb^+ + M\cdot\ (k_{-2}) \rightleftharpoons {}^{Fe}Hb + M\ (k_2)$. The equilibrium constant obtained from $([{}^{Co}Hb^+][{}^{Fe}Hb]/[{}^{Co}Hb][{}^{Fe}Hb^+])_{equil}$ is 5.9 ± 1.2; the value calculated from the redox potentials of the two proteins is 6.9. The rate constant k_1 was found to be 2.39 ± 0.26 M sec^{-1} and the value of k_{-2} is two to four times smaller. Cyanide and azide ions retard the Co → Fe transfer but enhance the Fe → Co transfer. Fluoride ion has no effect on these processes. Redox potentials for the $^{Fe}Hb/^{Fe}Hb^+$ couple in the presence of these ions have been measured. Their effects on electron transfer are a manifestation of the redox potentials. The role of the mediator appears to be one of transporting an electron from one protein to another.

The mechanism by which hemoproteins transfer electrons is of great biochemical interest. Enzymes such as catalases, reductases, peroxidases, cytochromes, and oxidases play crucial roles in these processes. There are extensive discussions regarding the electron transfer pathways involving cytochrome c. These discussions have intriguing elegance and detail rendered possible by the X-ray structures.[1-4] Two types of mechanisms are currently accepted: direct (e.g., cytochrome c-cytochrome oxidase) and indirect (e.g., DPNH-cytochrome reductase). On the analytical side, it has been known for some time that the rate of electrochemical equilibration of hemeproteins with electrodes is promoted by the addition of dye molecules of an appropriate redox potential.[5a] These dye molecules are referred to as mediators. Though mediators are extensively employed to assist the measurement of redox potentials of enzymes, their mode of function remains a mystery to the practitioner.

This paper is concerned with the kinetics and mechanism of electron transfer between hemoglobins and the involvement of mediators. The work stems from our broader effort in the study of the role of metal atom in determining the conformation and function of metalloenzymes. We have taken the direct approach of metal substitution in this investigation. The electronic structure of the prosthetic group in cobaltomyoglobin[6] and cobaltohemoglobin,[7] sometimes referred to as coboglobin,[8] has been studied with single crystal EPR spectroscopy.[6] The oxygen ligand was found to be π bonded to Co in oxycobaltomyoglobin and the movement of the Co atom associated with oxygenation and deoxygenation is concluded to be small. The biochemical properties of coboglobin have been compared with those of the native hemoglobin.[9] Cobalt derivatives of cytochrome c have also been prepared and characterized.[10] The redox potentials of the $^{Co}Hb/^{Co}Hb^+$ couple[11] have been determined as functions of pH with the aid of Methylene Blue and thionin as mediators.[9] The value of $E_{m,7}$ was found to be +0.100 V. This is compared with $E_{m,7}$ of +0.147 V for the $^{Fe}Hb/^{Fe}Hb^+$ couple.[9,12,13] Because of the closely similar redox potentials, it was thought that electron transfer results obtained between hemoglobin and coboglobin should model the redox process between the native species alone. The system offers a means to delineate the role of mediator without the intervention of electrodes. In this paper the electron transfers from ^{Co}Hb to $^{Fe}Hb^+$ (Co → Fe transfer) and from ^{Fe}Hb to $^{Co}Hb^+$ (Fe → Co transfer) are discussed.

Experimental Section

Materials. Adult human blood was used within a day of donation to prepare ^{Fe}Hb. The procedure is one of dilution lysis described by Perutz.[14] $^{Fe}Hb^+$ was prepared by treating $^{Fe}HbO_2$ with a twofold excess of potassium ferricyanide and passage through a G-25 Sephadex column. ^{Co}Hb and $^{Co}Hb^+$ were prepared as previously described.[9] The oxygenation curve of each batch was measured with an automatic apparatus similar to the one described by Imai et al.[15] Only preparations with Hill coefficient greater than 1.8 were used in these electron transfer studies. Methylene Blue chloride was obtained from Baker. NaCN, NaF, and NaN_3 are reagent grade chemicals.

Stock Solutions. All stock solutions of the proteins were about 0.284 mM in 0.1 M pH 7.13 sodium phosphate buffer (20°). Concentrations were determined as ^{Co}Hb or $^{Fe}Hb^+CN^-$ spectrophotometrically. Stock solutions were kept in crown-top pressure bottles (Lab glass, Vineland, N.J.) fitted with self-sealing neoprene liners and perforated metal caps for the insertion of syringe needles. Deoxygenation was accomplished by purging with water saturated prepurified nitrogen gas while the solution was maintained at 4° and gently agitated with a magnetic stirring bar. Four hours of flushing by this procedure was found adequate to remove all the dissolved oxygen as judged by the total absence of the $^{Co}HbO_2$ EPR signal[6] in a ^{Co}Hb solution.

A stock solution of Methylene Blue (9.05 mM) was also prepared in 0.1 M phosphate buffer and was used in all of the runs. This solution was deoxygenated with nitrogen bubbling for 1 hr. Solutions of NaCN, NaN_3, and NaF were similarly prepared.

For experiments at pH 9.0, sodium borate buffer was used to prepare the stock solutions. Otherwise, the procedure was the same as above.

Electron Transfer Experiments and EPR Measurement. Two types of reaction vessels were used to conduct the electron transfer experiments. The first was a 25 ml rubber stoppered Erlenmeyer flask fitted with glass inlet for argon or nitrogen and an outlet tube terminated with a small "Bunsen valve" type rubber gasket for the introduction of reactants via 2 mm o.d. glass tubing attached to syringes with silicone tubing. In this set-up the inert atmosphere was maintained by a slow leakage of the inert gas. The second type of reaction vessel was the crown-top bottle described in the previous section. In these experiments there was a positive pressure of inert gas and stainless steel needles (20 gauge) were used in all the transfers. The syringes used in these operations were "sealed" with water and rinsed with nitrogen for at least six cycles prior to its usage in transfer of solutions. Generally the total reaction volume was 4 ml.

The course of reaction was monitored by withdrawing a 0.3-ml aliquot of reaction mixture and introducing it into a nitrogen filled 4 mm quartz EPR cell. The sample was immediately frozen in liquid nitrogen and the time was recorded. The intensity of the $^{Fe}Hb^+$ (g = 6) and or ^{Co}Hb (g = 2.3) EPR signals was used to follow the reaction. A Varian E-9 X-band spectrometer was used in these measurements. In rare instances oxygen inadvertently leaked into the reaction vessel or EPR cell. This is readily seen by the appearance of the g = 2.0 signal of $^{Co}HbO_2$. In such cases, the experiment was discontinued and the results were discarded.

The following practice was adopted in order to obtain the great-

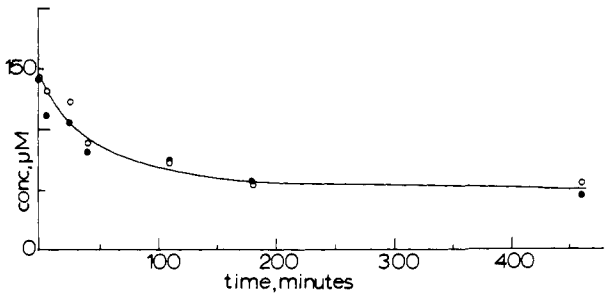

Figure 1. Plot of hemoglobin concentrations as metal ions versus time: (○) ^{Co}Hb; (●) $^{Fe}Hb^{+}$. Initial conditions: $[^{Co}Hb]_0 = [^{Fe}Hb^{+}]_0 = 0.141$ mM, $[M]_0 = 0.100$ mM (run no. 1).

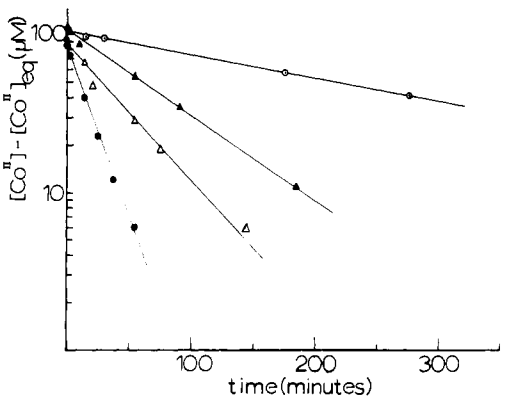

Figure 2. Rate of oxidation of ^{Co}Hb for varying Methylene Blue concentrations. Each experiment had initially 0.141 mM of ^{Co}Hb and $^{Fe}Hb^{+}$. [M]: (⊙) 0.024 mM, (▲) 0.1 mM; (△) 0.19 mM; (●) 0.39 mM.

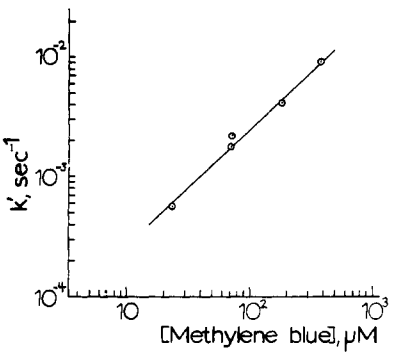

Figure 3. Log k' versus log [M]. The k''s are from the slopes in Figure 2. Also included is a duplicate run at 0.1 mM Methylene Blue.

est possible precision. For a given series of aliquots, the same EPR cell was used. It was cleaned with care after each measurement and returned with a new aliquot to the identical position and orientation in the liquid nitrogen dewar. The position of the dewar was not disturbed during a run. Also maintained constant are the microwave power, the klystron frequency, and the detector current. Even with this procedure, a fair amount of scatter exists in the data due probably to the noise level necessitated for measurement at low concentrations. The formation of ice particles in the liquid nitrogen dewar could also affect signal intensity.

Redox Potentials. Redox potentials of the $^{Fe}Hb/^{Fe}Hb^{+}$ couple were measured by the method previously described.[9] Equimolar mixtures of the oxidized and reduced deoxygenated proteins were measured to have a $E_{m,7} = +0.147$ V in agreement with other workers and our previous work.[9,12,13] The effect of anion was determined in each case by adding 10 μl of a concentrated solution of sodium salt to the above mixture to yield a fivefold excess of NaCN, NaN_3, or NaF over the hemoglobin concentration. Indigo Carmine ($E_{m,7} = -0.140$ V[5a]) was added as mediator at 0.02 mol fraction of the total iron concentration. A constant potential was reached within 20 min in all cases.

Data and Results

The experiments to be described below call for best possible preparations of coboglobins in order to assure maximum stability. The criteria are the $p_{0.5}$ and the Hill coefficient.[9] Results of experiments were discarded when there was noticeable denaturation of coboglobins. In addition, coboglobin is more easily autoxidized than the native protein. Thus rigorous exclusion of oxygen is essential to obtain meaningful results. Autoxidation is manifest when for instance there is more ^{Co}Hb oxidized than $^{Fe}Hb^{+}$ reduced.

When there is no denaturation or autoxidation, then no electron transfer between hemoglobin and coboglobin takes place in the absence of mediator as measured by changes in EPR spectra. Furthermore, numerous experiments established that at pH 7.13 Methylene Blue would neither reduce $^{Co}Hb^{+}$ or $^{Fe}Hb^{+}$ nor oxidize ^{Co}Hb or ^{Fe}Hb without the presence of the complementary species to accept or donate electrons, respectively. Electron transfer becomes possible when mediator is added to a mixture of a redox couple.

Co → Fe Transfer. A typical set of data at pH 7.13 and 25° is given in Figure 1. The reaction mixture is 0.141 mM in both ^{Co}Hb and $^{Fe}Hb^{+}$ and 0.1 mM in M^{+}, where M^{+} is the oxidized form of Methylene Blue. The intensities of both EPR signals decay monotonically to equilibrium values after several hundred minutes. This shows that ^{Co}Hb is oxidized and $^{Fe}Hb^{+}$ is reduced at the same rate. Plots of log ($[Co] - [Co]_{equil}$) versus time are linear, where $[Co]_{equil}$ is the equilibrium concentration of ^{Co}Hb reached after several hours. The rate was dependent on the mediator concentration. The results are summarized in Table I and the semilog plots of the reaction rates are shown in Figure 2. The equilibrium concentrations of ^{Co}Hb listed in column 3 are nearly independent of $[M]_0$ within experimental variations. Therefore, in the *presence of the acceptor* $^{Fe}Hb^{+}$, the mediator can be said not to enter into the net reaction.

From the slope of the lines in Figure 2 we obtained k_1' for the disappearance of ^{Co}Hb. That k_1' varies directly with [M] is shown by Figure 3 and the values of $k_1'' = k_1'/[M]_0$ in Table I. The equilibrium constants, tabulated in column 7, were calculated from the observed equilibrium concentrations of ^{Co}Hb and $^{Fe}Hb^{+}$.

The rate of reduction of $^{Fe}Hb^{+}$ was monitored by the $g = 6$ signal. In some of the experiments this rate was identical to that of the oxidation of ^{Co}Hb (see Figure 1, for example).

Table I. Dependence of Co → Fe Transfer on Mediator Concentration[a]

Run no.	$[M]_0$, mM	$[^{Co}Hb]_{equil}$, mM	$[^{Fe}Hb^{+}]_{equil}$, mM	k_1', 10^4 sec^{-1}	k_1'', (M sec)$^{-1}$	K
3	0.024	0.040	0.055	0.55	2.29	4.0
21	0.074	0.030	0.061	2.1	2.84	4.9
22	0.074	0.030	0.065	1.71	2.32	4.4
2	0.19	0.035	0.055	4.13	2.17	5.3
4	0.39	0.04	0.04	9.08	2.33	6.4

[a] $[^{Co}Hb]_0 = [^{Fe}Hb^{+}]_0 = 0.141$ mM; temp = 25°.

Table II. Dependence of Co → Fe Transfer on $^{Fe}Hb^+$ Concentration[a]

Run no.	$[^{Co}Hb]_o$, mM	$[^{Fe}Hb]_o$, mM	$[^{Co}Hb]_{equil}$, mM	$[^{Fe}Hb^+]_{equil}$, mM	k_1', 10^4 sec^{-1}	k_1'', (M sec)$^{-1}$	K
57	0.142	0.142	0.04	0.04	1.78	2.54	6.5
58	0.142	0.036	0.07	0.004	2.71	3.87	8.1
59	0.036	0.142	0.002	0.108	2.03	2.89	5.5
5	0.142	0.362	0.026	0.282	2.31	3.27	1.2

[a] $[M]_o$ = 0.07 mM; temp = 25°.

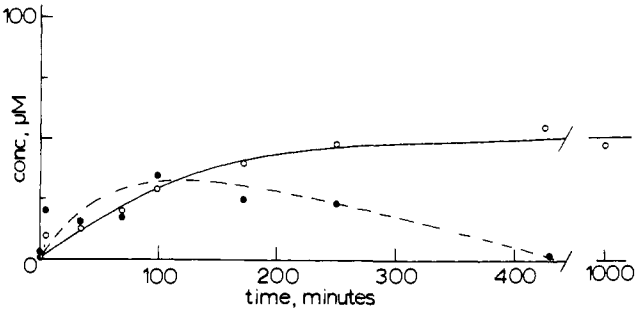

Figure 4. Plot of hemoglobin concentrations as metal ions versus time: (○) ^{Co}Hb; (●) $^{Fe}Hb^+$. Initial conditions: $[^{Co}Hb^+]_0 = [^{Fe}Hb]_0 = 0.141$ mM; $[M]_0 = 0.074$ mM (run no. 7). Note difference in ordinate scale in comparison with Figure 1.

In a number of other experiments the initial rates of the two reactions are the same, but after 50 to 100 min, the rate for $^{Fe}Hb^+$ slowed down so that its equilibrium concentration is somewhat higher than that of $[^{Co}Hb]_{equil}$. Even though the measurement of $[^{Fe}Hb^+]$ is less accurate than that of $[^{Co}Hb]$ because the $g = 6$ signal is broader and lower in signal to noise ratio, the observed difference is definitely greater than the experimental error.

The dependence of the rate of oxidation of ^{Co}Hb on $[^{Fe}Hb^+]$ was investigated at $[^{Co}Hb]_0 = 0.142$ mM, $[M]_0 = 0.07$ mM, and $[^{Fe}Hb^+] = 0.036$–0.36 mM. The results are summarized in Table II. The values of k_1'' are generally on the high side as compared to those in Table I. However, it can be said that tenfold changes in $[^{Fe}Hb^+]$ did not cause significant variation in k_1''. There is no trend of dependence of k_1 on $[^{Fe}Hb^+]_0$. We can now express the rate law for the Methylene Blue mediated Co → Fe transfer in hemoglobins as

$$-\frac{d([^{Co}Hb] - [^{Co}Hb]_{equil})}{dt} = (2.39 \pm 0.26)([^{Co}Hb] - [^{Co}Hb]_{equil})[M]_0 \quad (1)$$

Several experiments were performed at 0°. No change in ^{Co}Hb or $^{Fe}Hb^+$ intensity was observed over a period of 6.5 hr. The Co → Fe transfer apparently has a significant activation energy.

Fe → Co Transfer. When stock solutions of ^{Fe}Hb, $^{Co}Hb^+$, and Methylene Blue are mixed, EPR signals at $g = 6$ and 2.3 develop indicating the occurrence of electron transfer processes. An example is shown in Figure 4. The ^{Co}Hb signal increases monotonically and approaches an equilibrium concentration which is the same as that found in the Co → Fe transfer (compare Figures 1 and 4). There is no simple kinetic plot for the Fe → Co transfer (vide infra). However, the apparent rate constant k_{-2} can be estimated from the initial rate, $k_{-2} \approx$ (initial rate)/$[^{Fe}Hb]_0[M]_0$. The results are summarized in Table III.

The $g = 6$ signal showed large scatter during the early stage of the reaction and reached a maximum value between 50 and 150 min depending upon the particular experiment. Subsequently, the $g = 6$ signal decreases to nearly zero. This behavior can be explained by product inhibition for the oxidation of ^{Fe}Hb.

Table III. Fe → Co Transfer Reactions at 25°

Run no.	$[^{Fe}Hb]_o$, mM	$[^{Co}Hb^+]_o$, mM	$[M]_o$, mM	Initial rate, 10^8 M sec^{-1}	k_{-2}, (M sec)$^{-1}$
7	0.141	0.141	0.073	0.62	0.60
9	0.141	0.141	0.145	2.95	1.45
23	0.142	0.142	0.074	0.54	0.54

Effect of Anions. The effect of CN^-, N_3^-, and F^- on the electron transfer was studied. The first two ions have a strong inhibitory effect on the Co → Fe redox process whereas the third has virtually no effect. In the former reactions, there were not sufficient changes in the EPR signal intensities to merit rate analysis, instead only the initial rates, R_i, were given in Table IV. The rate constants k_1 obtained with fluoride ion (column 7) compare favorably with those reported in Table I. The presence of cyanide or azide ion reduces the initial rate by a factor of between 2 and 8. The $[^{Co}Hb]_{equil}$ was much higher with these added ions. The quantity of ^{Co}Hb oxidized at equilibrium is ½ to 1/10 of the amount reacted in the presence of fluoride ion.

The CN^- and N_3^- appear to drive the Fe → Co transfer to completion. This is illustrated by Figure 5. This figure is to be compared with Figure 4; the two experiments differ only in the presence of 0.7 mM of N_3^- in the former. Similar behavior was seen in the presence of CN^- ions. In these experiments nearly all of the $^{Co}Hb^+$ was reduced.

Evidence for Free Radical Species. It is reasonable to expect that methylene radical ion would be an intermediate in the reactions studied here. The EPR spectra of Methylene Blue radical ion have been briefly described by Heineken et al.[16] We reproduced their room temperature results. In neat H_2SO_4, we obtained a 1:2:2:1 spectra with line separation of 6.7 G at room temperature. This can be assigned to the protonated radical ion. In alkaline methanol, the room temperature EPR spectrum is a poorly resolved triplet of the radical ion. A signal can be observed at high gain for a concentrated (0.1 M) solution of Methylene Blue in pH 7.13 phosphate buffer; it appears to be a combination of the other two signals. There could be an equilibrium between protonated and unprotonated radical ions.

At 77°K, both the protonated and unprotonated radical ion gave a single symmetric line with $g = 2.005$ and a width of 6.5 and 10 G, respectively.

Organic free radicals were detected only in some of the experiments. When a signal is seen, the radical concentration amounts to about 5–10% of the Methylene Blue. There are differences in the spectra in various experiments. In run 60 with $[^{Co}Hb^+]_0 = 0.14$ mM, $[^{Fe}Hb] = 0.14$ mM, $[M]_0 = 0.07$ mM, and $[CN^-] = 0.7$ mM, the signal is a single symmetric line having $g = 2.0037$ and a width of ca. 17 G. In run 5 (Table II), there was a similar signal except with a hint of hyperfine features. In other experiments, the epr spectra can be best interpreted as a combination of the M· signal and another one with a g value of 2.016. The latter could be a sulfur radical. The g value of alkyl sulfide radical has been reported to be 2.014.[17] A more likely assignment is a peroxy radical. The g values of peroxy radicals range from 2.0113 to 2.0160.[18] If this is true, then the appearance of the $g = 2.016$ signal suggests the experiment to

Table IV. Co → Fe Transfer in the Presence of Anions[a]

Run no.	Anion	Δ[CoHb], m*M*	Δ[FeHb⁺], m*M*	R_i, 10^8 (M sec)$^{-1}$	k_1', 10^4 sec^{-1}	k_1'', (M sec)$^{-1}$
24	CN^-	0.04	*b*	0.54		
61	CN^-	0.023	*b*	0.40		
27	N_3^-	0.01	~0	0.19		
64	N_3^-	0.04	0.04	0.64		
26	F^-	0.11	0.075	1.3	1.86	2.48
63	F^-	0.10	0.10	1.5	1.44	1.95

[a] $[CoHb]_0 = [FeHb^+]_0 = 0.142$ m*M*; $[M]_0 = 0.074$ m*M*; 25°. [b] $FeHb^+CN^-$ does not have an EPR signal at 77°K.

be slightly contaminated by oxygen. This might also explain the absence of M· signal and differences in amount of Co → Fe and Fe → Co transfer in some cases.

When a M· radical signal is seen, its intensity increases during the course of reaction reaching a maximum value at the time equilibrium is established. Afterward the intensity remains relatively constant in some experiments and shows a slow decline in others.

Discussion of Results

There are two plausible mechanisms for the electron transfer between hemoglobin and coboglobin as mediated by Methylene Blue. The first mechanism invokes two bimolecular equilibria, viz.,

$$^{Co}Hb + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} {}^{Co}Hb^+ + M\cdot \quad (2)$$

$$^{Fe}Hb^+ + M\cdot \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {}^{Fe}Hb + M \quad (3)$$

with the mediator acting as a real electron carrier. A second mechanism postulates a bridging complex, i.e.,

$$^{Co}Hb + M \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} {}^{Co}HbM \quad (4)$$

$$^{Co}HbM + {}^{Fe}Hb^+ \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} {}^{Co}HbM^{Fe}Hb^+ \quad (5)$$

$$^{Co}HbM^{Fe}Hb^+ \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} {}^{Co}Hb^+ + {}^{Fe}HbM \quad (6)$$

$$^{Fe}HbM \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} {}^{Fe}Hb + M \quad (7)$$

In this case the mediator is thought to provide a conducting pathway for the electron. The fact that Methylene Blue radical signal was observed in some of the experiments tends to support the first mechanism. Gel permeation fails to provide evidence of any complex formation between the protein and Methylene Blue. Finally, this simpler mechanism appears adequate in accounting for both quantitative and semiquantitative results given above.

If we adopt the first mechanism, then the equilibrium constant can be calculated from the known redox potentials of the two proteins (vide supra).[9] Substituting these values into

$$(RT/F)\ln K = E_{m,7}^{Fe} - E_{m,7}^{Co} \quad (8)$$

we obtain $K = 6.9$. The average value of K for the results in Tables I and II is 5.9 ± 1.2. The value for run 5 was not included in this average, it is anomalously small for some unidentified reason.

The rates of the reactions according to the first mechanism, assuming a steady state for M·, are given by

$$-\frac{d[^{Co}Hb]}{dt} = \frac{d[^{Co}Hb^+]}{dt} = \left\{W_1 - \frac{W_1(W_1 + W_{-2})}{(W_{-1} + W_2)}\right\}[M] \quad (9)$$

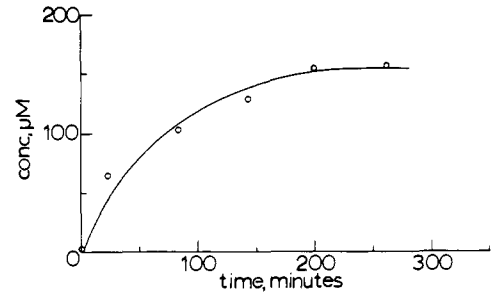

Figure 5. Effect of azide ion of Fe → Co transfer. $[^{Co}Hb^+]_0 = [^{Fe}Hb]_0 = 0.141$ m*M*; $[M]_0 = 0.074$ m*M*; $[N_3^-] = 0.71$ m*M*.

$$-\frac{d[^{Fe}Hb^+]}{dt} = \frac{d[^{Fe}Hb]}{dt} = \left\{\frac{W_2(W_1 + W_{-2})}{(W_{-1} + W_2)} - W_{-2}\right\}[M] \quad (10)$$

where $W_1 = k_1[^{Co}Hb]$, $W_{-1} = k_{-1}[^{Co}Hb^+]$, $W_2 = k_2[^{Fe}Hb^+]$, and $W_{-2} = k_{-2}[^{Fe}Hb]$. Several factors contribute toward the relative ease of these reactions. Electronic consideration would assign k_{-1} the smallest values. An electron is being introduced into the antibonding e_g orbital. The crystal field activation energy is estimated to be ca. 4 kcal mol^{-1}. The reverse reaction of oxidation of ^{Co}Hb is probably more favorable, even though changes in metal ligand bond length probably accompany the process. The presumed unfavorable rehybridization of the complex is partly countered by the favorable removal of an antibonding electron. Both k_2 and k_{-2} should be greater than k_1 and k_{-1}. Addition or removal of an electron from a t_{2g} orbital does not require any disruption of the hybridization of the complex, there is no crystal field activation energy. The reduction of $^{Fe}Hb^+$ is probably much faster than the reverse process for the following reasons. The Fe atom is about 0.7 to 0.95 Å out of the heme plane in deoxyhemoglobin,[19] whereas this distance is reduced to 0.3 Å in methemoglobin.[19] Orbital overlap between the $d\pi$ orbitals of Fe and the $p\pi$ orbitals of porphyrin should be more favorable in methemoglobin. If the electron is transferred via the ligand π system as it will be proposed below, k_2 should be greater than k_{-2}. Furthermore, ^{Fe}Hb has tighter tertiary and quaternary conformation than $^{Fe}Hb^+$. This is shown both by X-ray diffraction and by the faster rate of reaction of $^{Fe}Hb^+$ with pMB[11] and ^{Fe}Hb for the same reaction.[9,20] These last two orbital overlap and steric considerations tend to favor the cobalt species. EPR results of single crystal ^{Co}Mb and $^{Co}MbO_2$[6] and X-ray results on model cobalt porphyrins[21-23] showed that the cobalt atoms are nearly in plane in all the compounds. Furthermore, the environment surrounding the "heme" in the cobalt species appears to be more accessible than that in the native enzyme as judged by the rates of pMB reaction.[9]

The above suggests that $k_2 \gg k_{-1}, k_{-2}$. We can now obtain solutions to eq 9. Let $[^{Co}Hb]_0 = a$, $[^{Fe}Hb^+]_0 = b$, $[^{Co}Hb^+] = x$, and assume M· to reach stationary concen-

tration soon after mixing so $[M] = g[M]_0$ and $[M\cdot] = (1 - g)[M]_0$, then for $k_2 \gg k_{-1}, k_{-2}$, eq 9 reduces to

$$\frac{dx}{g[M]_0 dt} = k_1(a - x) - k_{-1}'\frac{x(a - x)}{(b - x)} \quad (11)$$

where $k_{-1}' = k_1 k_{-1}/k_2$. For the experiments in Table I, $a = b$, therefore

$$\frac{dx}{g[M]_0 dt} = k_1(a - x) - k_{-1}'x \quad (12)$$

At equilibrium

$$k_1(a - x_{equil}) = k_{-1}' x_{equil} \quad (13)$$

Combining eq 12 and 13 gives

$$\frac{dx}{g[M]_0 dt} = \frac{k_1 a(x_{equil} - x)}{x_{equil}} \quad (14)$$

Substituting the definitions of x, a, and x_{equil} into eq 14 gives

$$-\frac{d([^{Co}Hb] - [^{Co}Hb]_{equil})}{dt} = gk_1(1 + K^{-1/2})([^{Co}Hb] - [^{Co}Hb]_{equil})[M]_0 \quad (15)$$

Its solution is

$$[^{Co}Hb] - [^{Co}Hb]_{equil} = ([^{Co}Hb]_0 - [^{Co}Hb]_{equil}) \exp\{-gk_1(1 + K^{-1/2})[M]_0 t\} \quad (16)$$

Comparison of eq 1 and 15 showed that they are equivalent and that $k_1'' = g(1 + K^{-1/2})k_1$.

The case of unequal $[^{Co}Hb]$ and $[^{Fe}Hb^+]$ is more complicated. If we let $(k_1 + k_{-1}') = \alpha$, $(\alpha a + k_1 b) = \beta$, $k_1 ab = \gamma$, and $Q^2 = \beta^2 - 4\alpha\gamma$, then the solution to eq 11 is

$$\frac{b - (\beta/2\alpha)}{Q}\ln\frac{(2\alpha x - \beta - Q)(\beta - Q)}{(\beta + Q)(2\alpha x - \beta + Q)} - \frac{1}{2\alpha}\ln\frac{\alpha x^2 - \beta x + \gamma}{\gamma} = g[M]_0 t \quad (17)$$

This complicated solution is not too useful in the analysis of our data. However, it can be used to show that the rate of Co → Fe transfer should not be sensitive to $[^{Fe}Hb^+]_0$. The approximation is that $k_1 \gg k_{-1}'$ which follows directly from our earlier assumption of $k_2 \gg k_{-1}$. It can be shown that eq 17 is simplified to

$$[^{Co}Hb] - [^{Co}Hb]_{equil} = [^{Co}Hb]_0 \exp(-k_1 g[M]_0 t) - [^{Co}Hb]_{equil} \quad (18)$$

Addition of cyanide or azide ion should not significantly affect the redox reaction of ^{Co}Hb since the oxidized protein has no affinity for N_3^- and only weak affinity for CN^-. This is, however, not true for $^{Fe}Hb^+$ which has strong affinity for these ions. The redox potentials, $E_{m,7}$ for the $^{Fe}Hb/^{Fe}Hb^+$ system under the conditions of fivefold excess of CN^-, N_3^-, and F^-, are −0.008, +0.050, and +0.147 V, respectively. The value for CN^- is about 0.040 V lower than that of previous workers[24] who found a dependence of potential on CN^- concentration. However, the measurements were performed without the benefit of a mediator. Using the above $E_{m,7}$'s and assuming that the $E_{m,7}$ for the $^{Co}Hb/^{Co}Hb^+$ couple is not altered by these anions, we calculated equilibrium constants for $[^{Co}Hb^+][^{Fe}Hb]/[^{Co}Hb]\cdot[^{Fe}Hb^+X]$ to be 0.016, 0.15, and 6.8 for X = CN^-, N_3^-, and F^-, respectively. It is clear that the presence of fluoride ion has no significant effect upon the redox potential and thus no effect on electron transfer in agreement with observation. Since the redox potentials for the $^{Fe}Hb/^{Fe}Hb^+X^-$ are much lower than the $^{Fe}Hb/^{Fe}Hb^+$ potential when X^- is CN^- or N_3^-, the equilibrium constants for the cobalt-iron electron exchange are shifted by factors of 420 and 46, respectively. Consequently, the Fe → Co transfer is driven to completion whereas the Co → Fe transfer is strongly inhibited by CN^- and N_3^-.

Table V. Kinetic and Equilibrium Constants for the Reaction of Methemoglobin with Anionic Ligands

Ligand	pH	Temp, °C	pK'	k_{off}, sec^{-1}	Ref
CN^-	7.0	20	8.9	1.3×10^{-7} [a]	24, 25
N_3^-	7.5	25	5.41	2.8×10^{-3}	26, 27
F^-	7.0	20	1.9	5×10^{-2}	24, 25

[a] Calculated from k_{on} at pH 6.05.[28]

The electron transfer in this system probably proceed via an outersphere pathway. Innersphere mechanism is unlikely for at least two reasons. There is insufficient space in the heme pocket to accommodate a Methylene Blue molecule.[19] Furthermore, in the presence of CN^- ion, innersphere transfer has to be preceded by the dissociation of the complex. The off rate constants for $^{Fe}Hb^+X^-$ are given in Table V as well as the stability constants K'. The value of k_{off} for $^{Fe}Hb^+CN^-$ is so small that there will be no measureable rate of transfer if the process has to be preceded by the dissociation of the cyanide complex.

We envisualize an outersphere transfer mechanism involving a transition state complex with the planes of the heme and of the Methylene Blue side by side and coplanar for maximum overlap of the π orbitals. The process is analogous to the electron transfer involving bridging ligands which has been thoroughly investigated by Taube and coworkers. An example is the reduction of fumaratopentaamminocobalt(III) by chromous ion.[30] The intermediary of radical ion in electron transfer also has ample precedents. An example is the oxidation of formatopentaamminocobalt(II) by permanganate ions.[31]

Acknowledgment. This research was supported by the National Institute of Health under Grants No. HL-14270 and AM 14779.

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(11) Abbreviations: ^{Co}Hb, cobaltohemoglobin; $^{Co}Hb^+$, cobaltihemoglobin; $^{Fe}Hb_A$, ferrohemoglobin; $^{Fe}Hb_A^+$, ferrihemoglobin; M, Methylene Blue chloride; Mb, myoglobin; pMB, *p*-chloromercuribenzoate.
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Reversible Reactions of Gaseous Ions. IX. The Stability of C_4–C_7 Tertiary Alkyl Carbonium Ions

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Received June 25, 1974

Abstract: The hydride transfer equilibria $t\text{-}C_4H_9^+ + RH \rightleftharpoons R^+ + i\text{-}C_4H_{10}$ where R^+ = 2-methylpentyl ion (2-MP$^+$), 3-MP$^+$, 2,3-DMB$^+$, and 2,4-DMP$^+$ have been investigated by pulsed electron beam high pressure mass spectrometry. The equilibrium constants for the reactions and the temperature coefficients of the equilibrium constants over the range 334–610°K have been used to establish a network of thermodynamic quantities for the reactions. The ionic enthalpies determined (relative to $\Delta H_f°$ (*tert*-butyl) = 169.1 kcal/mol as standard) are believed to be accurate to 0.2 kcal/mol with $\Delta H_f°$(2-MP$^+$) = 155.6, $\Delta H_f°$(3-MP$^+$) = 155.3, $\Delta H_f°$(2,3-DMB$^+$) = 153.2, and $\Delta H_f°$(2,4-DMP$^+$) = 134.9 kcal/mol. Trends in stability have been related to structural and ionic factors. The entropies for the reactions of *tert*-butyl ion with the three isomeric tertiary hexanes (2-MP, 3-MP, and 2,3-DMB) vary between 0 and +2 eu. The entropy change of −29.4 eu for the reaction of *tert*-butyl ion with 2,4-DMP suggests that the $C_7H_{15}^+$ ion formed in this reaction is a highly constrained species.

We recently[1] reported an investigation of hydride transfer equilibrium in the $t\text{-}C_4H_9^+$ ($i\text{-}C_5H_{12}$, $i\text{-}C_4H_{10}$) $t\text{-}C_5H_{11}^+$ system using the technique of pulsed electron beam high pressure mass spectrometry. Equilibrium constants, free energies, enthalpies, and entropies were determined for the reaction

$$t\text{-}C_4H_9^+ + i\text{-}C_5H_{12} \rightleftharpoons t\text{-}C_5H_{11}^+ + i\text{-}C_4H_{10} \quad (1)$$

In the present study we report the results of our investigations of the hydride transfer reactions of *tert*-butyl ions with the three isomeric tertiary hexanes, namely

$$t\text{-}C_4H_9^+ + 2\text{-MP} \rightleftharpoons 2\text{-MP}^+ + i\text{-}C_4H_{10} \quad (2)$$

$$t\text{-}C_4H_9^+ + 3\text{-MP} \rightleftharpoons 3\text{-MP}^+ + i\text{-}C_4H_{10} \quad (3)$$

$$t\text{-}C_4H_9^+ + 2,3\text{-DMB} \rightleftharpoons 2,3\text{-DMB}^+ + i\text{-}C_4H_{10} \quad (4)$$

and our investigation of the hydride transfer equilibrium occurring between *tert*-butyl ions and a tertiary heptane

$$t\text{-}C_4H_9^+ + 2,4\text{-DMP} \rightleftharpoons C_7H_{15}^+ + i\text{-}C_4H_{10} \quad (5)$$

These investigations are being undertaken to establish a network of relative thermodynamic quantities for gaseous carbonium ions. Obtaining accurate thermochemical values will permit the evaluation of the effects of structural variations upon intrinsic carbonium ion stabilities.

Experimental Section

These measurements were made on the Rockefeller Chemical Physics mass spectrometer operated in the pulsed electron beam mode of ionization. The apparatus and experimental technique were identical with those described previously,[1,3,4] except that here the instrument was operated under field-free conditions with the repeller maintained at ion chamber potential. Gas from a mixture of known composition ($i\text{-}C_4H_{10}$/*tert*-alkane) was admitted into the ion source. The ion intensities of the *tert*-butyl ion (*m/e* 57) and the tertiary alkyl ions were monitored at variable reaction times at constant temperatures and pressures. The source pressure was adjusted at each temperature to keep the total number density constant ($N_T \simeq 4.0 \times 10^{16}$ molecules/cm^3). A typical pulsing sequence consisted of the 600 eV electron beam pulsing on for 100 μsec initiating ion production. Ions leaving the source are defocused except for the duration of the ion focus pulse, typically 100 μsec, which occurred at variable delay times (200–800 μsec) after the electron beam "on" pulse.

The reagents used were Matheson Instrument grade (99.5%) isobutane, Matheson Coleman and Bell Chromatoquality grade (99+ mol %) 2-MP, 3-MP, and 2,4-DMP, and K & K 2,3-DMB. Purities were checked by gas chromatography and found to be better than 99%.

Results and Discussion

The equilibrium constant for a general hydride transfer reaction $R_1^+ + R_2H \rightleftharpoons R_2^+ + R_1H$ is given by

$$K = \frac{[R_2^+][R_1H]}{[R_1^+][R_2H]} = \left(\frac{I_{R_2^+}}{I_{R_1^+}}\right)_{eq} \frac{P_{R_1H}}{P_{R_2H}} \quad (6)$$

We can examine the approach to equilibrium by measuring the ion intensity ratio ($I_{R_2^+}/I_{R_1^+}$) as a function of reaction time. In Figure 1 we show a sample plot of the apparent equilibrium constant, $K_a = (I_{99}/I_{57})(P_{i\text{-}C_4H_{10}}/P_{2,4\text{-DMP}})$ vs. reaction time for reaction 5. K_a initially rises and begins to level off above 300 μsec. Equilibrium has been achieved in the time independent region of K_a. Analogous plots were obtained for reactions 2–4 at several temperatures. van't Hoff plots were constructed from the values of the equilibrium constants in the plateau regions of the plots of K_a vs. reaction time. A typical van't Hoff plot obtained in this manner for one of the (*tert*-butyl, *tert*-hexyl) equilibria is shown in Figure 2 (upper). The temperature and pressure regimes employed for the (C_4, C_6) equilibria studies are 334–610°K and 1.4–2.5 Torr, respectively.

In our previous[1] experiments with the pulsed ionization high pressure technique we found that thermodynamic parameters obtained for reaction 1 by the continuous ioniza-