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Kinetics of CO Binding to Manganese, Zinc, and Cobalt Hybrid Hemoglobins

Sir:

We present a comparison of ligand binding by mixed-metal hybrid hemoglobins in which the iron atoms of one pair of subunits have been replaced by divalent Mn, Zn, or Co.[1] The noniron subunits of a hybrid do not bind CO, and only with cobalt is O_2 bound even weakly. Thus, through appropriate choice of metal hybrid and ligand, one can study the sequential binding of the "first two" ligands by the ferrous-iron chains while the analogous chains remain unliganded. The measurements permit us to characterize the individual chains within the hemoglobin A tetramer, as well as the allosteric equilibrium between the low-affinity (T) and high-affinity (R) forms of partially ligated hemoglobin A intermediates. These characterizations are unavailable from studies with hemoglobin A because of the strongly cooperative ligation process.[2] The use of a series of hybrids, involving several metals for which the metalloporphyrin properties are documented, further allows us to directly examine the influence of well-defined stereochemical changes in the prosthetic group of one chain pair on the T-R equilibrium and on the ligation properties of the complementary chains.

Hybrids were prepared by adaptations[3] of the scheme of Yip et al[4] or of Lee,[5] and their purities were confirmed by isoelectric focusing. Samples for kinetic measurements were typically ~5 μM in heme in 0.01 M bis-Tris-HCl, pH 6.6, containing ~0.1% β-mercaptoethanol. The (Co, Fe) hybrids were studied in 0.1 M KP_i buffer, pH 7, and therefore the (Mn, Fe) and (Zn, Fe) hybrids were also examined in this buffer for comparison. The manganese hybrids as synthesized are in the [(Mn_2(III), $Fe_2(CO)_2$] form. The Mn(II) form was prepared by addition of minimal dithionite following deaeration; methylene blue was used as a redox mediator. Descriptions are given elsewhere for the apparatus employed in flash photolysis at Northwestern[6] and at Cornell,[7] and for the stopped-flow measurements.[8]

Stopped-flow measurements reveal that the time course for CO binding to the Fe(II) chains of the unliganded (Mn, Fe) and (Zn, Fe) hybrids is homogeneous and pseudo first order in both the absence and the presence of inositol hexaphosphate (IHP). The low values for the rate constants (Table I) and the homogeneous time course indicate that each of these four unliganded hybrids is in the T state, confirming the earlier (Mn, Fe) observations.[9] In the absence of IHP, the Fe chains of the (α_2^{Fe},β_2^{M}) and (α_2^{M},β_2^{Fe}) hybrids have equal binding rates, indicating that in the T state the α and β chains have essentially equal reactivity toward carbon monoxide. Addition of IHP reduces the β chain binding rates by a factor of roughly two but does not influence the α rates; in conjunction with the flash photolysis results presented below, this demonstrates a tertiary structure influence on the reactivity within the T state.

The (Co, Fe) hybrids differ in that the time course for CO binding is not homogeneous. There is an initial rapid phase whose percentage decreases upon addition of IHP (Table I). In both cases, the rate constant for the slow component in a (Co, Fe)

Table I. CO Binding to Fe Chains of (Fe, M) Hybrid Hemoglobins[a]

hybrid	binding rates,[b] $\times 10^{-6}$ M^{-1} s^{-1} k_T −IHP	k_T +IHP	k_R	slow phase, %[c] −IHP	+IHP[d]	method
$\alpha^{Fe}\beta^{Mn}$	0.15	0.11	5.0	100	100	SF
				74	100	FP
$\alpha^{Mn}\beta^{Fe}$	0.14	0.05	6.0	100	100	SF
				70 (89[e])	100	FP
$\alpha^{Fe}\beta^{Zn}$	0.10	0.10	4.0	100	100	SF
				48	100	FP
$\alpha^{Zn}\beta^{Fe}$	0.10	0.05	4.5	100	100	SF
				58 (92[e])	100	FP
$\alpha^{Fe}\beta^{Co}$ [e]	0.09	0.09	6.0	85	~100	SF
				25	40	FP
$\alpha^{Co}\beta^{Fe}$	0.10	0.05	6.0	90	100	SF
				41	60	FP

[a] Conditions, except as noted: 0.01 M, bis-Tris-HCl, pH 6.6; T = 21 °C. The addition of IHP to 10-50 μM is indicated in "+IHP". [b] Estimated uncertainties: k_T, ±0.02 × 10^6 M^{-1} s^{-1}, k_R, ±0.5 × 10^6 M^{-1} s^{-1}. [c] Percentages are reproducible between samples to within ±5%. SF = stopped flow. FP = flash photolysis. [d] With some samples, small (≲5%) variable amounts of rapidly reacting materials are seen in the stopped-flow method, even with IHP present. This portion of the material is assumed to be partially denatured protein. [e] Conditions: 0.1 M KP, pH 7.0.

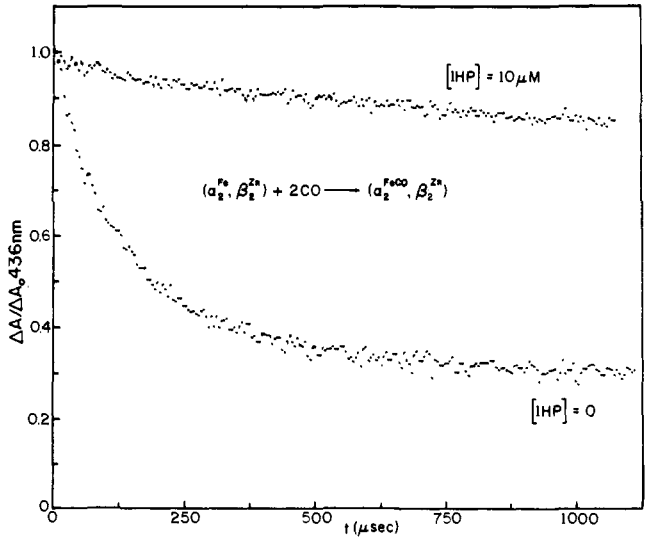

Figure 1. Recombination of CO with (α_2^{Fe}, β_2^{Zn}) monitored at 436 nm after flash photolysis of [$\alpha_2^{Fe}(CO)_2$,β_2^{Zn}]. Lower trace; [IHP] = 0, [CO] = 1.03 mM. Fitting a two-exponential decay to this trace gives percentages and rates as given in Table I. Upper trace; same conditions, but with [IHP] = 10 μM. The slow phase now represents the totality of the progress curve.

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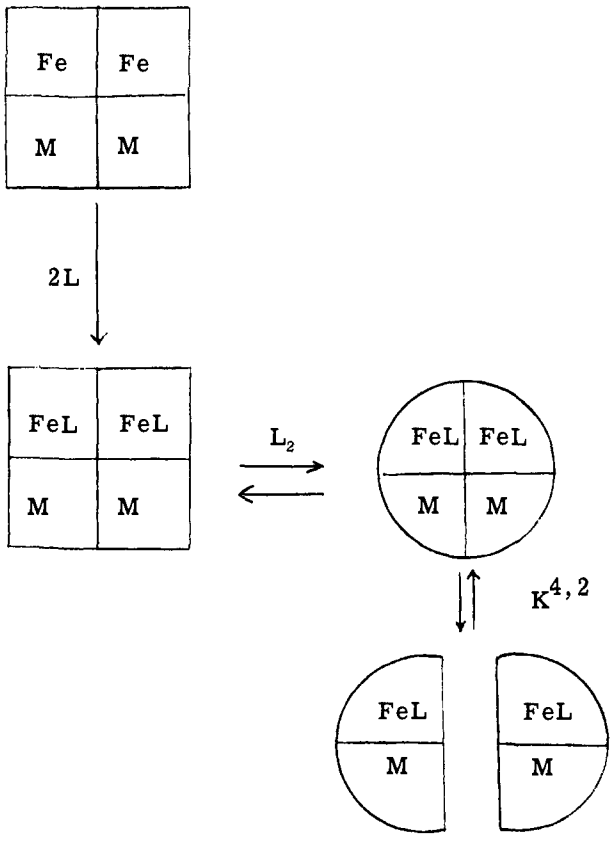
Scheme I



hybrid is the same as those for the corresponding Zn and Mn hybrids (Table I).

Upon full flash photodissociation of either of the $[Zn_2,Fe_2(CO)_2]$ or $[Mn_2,Fe_2(CO)_2]$ hybrids in the presence of IHP, the time course for CO rebinding (pH 6.6) is homogeneous (Figure 1) and pseudo first order. The second-order rate constants are low, characteristic of the T state,[10] and, within experimental error, are equal to those obtained in stopped-flow measurements with the corresponding unliganded proteins. Without IHP, CO rebinding after full flash photolysis of the diliganded hybrids is not homogeneous but is biphasic (Figure 1). The binding rate for the slow component is that observed in the stopped-flow experiments while the fast component binds with a rate roughly 40-fold higher (Table I). The proportion of slow component increases as the ionic strength is raised, and differences between oppositely substituted hybrids are not large (Table I).

The unusual behavior of the (Co, Fe) hybrids is strikingly evidenced in flash photolysis: even after addition of IHP, the CO rebinding is biphasic, and the rapidly rebinding component is large (Table I). However, the rate constants of the two phases are again essentially those of the (Mn, Fe) and (Zn, Fe) proteins (Table I).

The results for the (Mn, Fe) and (Zn, Fe) hybrids may be interpreted within the two-state model for cooperativity[11,2a] through Scheme I, in which a square represents the T and a circle the R conformations. The properties of Scheme I are as follows. Unliganded and singly liganded hybrids are fully in the T state, as expressed by the inequalities $[T]_0/[R]_0 \equiv L_0 \gg 1$, $[T]_1/[R]_1 \equiv L_1 \gg 1$, and the T state binds CO with a low rate ($\sim 0.1\ \mu M^{-1}$).[1] However, the binding of a second ligand sufficiently destabilizes the T state that one must explicitly consider the allosteric equilibrium between the T_2 and R_2 quaternary structures: $L_2 = [T]_2/[R]_2$. As a further complication, the T form of the protein is fully tetrameric, but the R form is in equilibrium with the symmetrical dimers (dissociation constant $K^{4,2}$). The addition of IHP stabilizes the T state relative to R and reduces the dissociation of R into dimers.[2]

Stopped-flow measurements begin with the tetrameric, T-state protein and give the kinetic characteristics of this state. Flash photolysis experiments begin with the diliganded protein equilibrium composition just described. Since the R → T switch is fast compared to the CO binding rates,[12] any tetramer which loses a ligand through photolysis, whether initially T_2 or R_2, rapidly becomes T and subsequently rebinds CO with the slow, T-state, rate. Therefore, the slowly rebinding component represents all the protein in the tetrameric state prior to photolysis: $[T]_2 + [R]_2$. However, dimers cannot associate into tetramers during the CO recombination period, and an unliganded Fe chain generated in a dimer binds CO with a fast, R-state, rate.[12] Therefore, the rapid component represents dimers. Since the presence of dimers requires a finite value for $[R]_2$, the rapid phase cannot be sizable unless L_2 is of the order of 10^1–10^0 or less. A quantitative treatment in terms of L_2 and $K^{4,2}$ is detailed elsewhere;[3] however, one particularly important feature will be mentioned here. Since $K^{4,2}$ is a characteristic of the R quaternary state and in first approximation is taken as independent of the extent of ligation,[2] it is likely to be comparably insensitive to metal substitution; it follows that for any two of our systems studied (at equal concentrations), the one with the larger slow component has the larger value of L_2.

It has been shown that manganese hemoglobin functions as a faithful analogue to the native protein.[1,7] Thus, the properties of the individual chains within the tetramer, and of the T → R equilibrium as observed with the (Mn, Fe) hybrids, can be directly applied to HbA. The biphasic kinetics observed upon photolysis of these hybrids indicate[3] that finite concentrations of dimers, and therefore of the R quaternary conformation, occur in solutions of diliganded hemoglobin intermediates; namely, L_2 is of order unity. The CO binding rates to the α and β chains in T-state Hb do not differ in the absence of IHP, but are ~twofold different in its presence (Table I).

The properties of the (Zn, Fe) hybrids are similar to those of (Mn, Fe). The Fe chains in the T-state tetramers bind CO with the same rates (Table I). Biphasic flash photolysis results show that the values of L_2 for the $[Zn_2, Fe_2(CO)_2]$ diliganded hybrids are again of order unity although the smaller slow component (Table I) indicates a slight lowering of L_2 (shift of the T ↔ R equilibrium toward R) with zinc.

These results lead to several conclusions about the properties of HbA. First, the rates of CO binding to individual chains are well established (Table I). Second, the properties of diliganded HbA intermediates can be quite well defined by comparing the results for the (Mn, Fe) and (Zn, Fe) hybrids. The structures of the five-coordinate M(II) porphyrins can be arranged in a sequence, with Fe(II) bracketed between Mn(II) and Zn(II). For example, in model compounds, the distance from the nitrogen of an axial imidazole to the center of the mean plane of the pyrrole nitrogens decreases in the order Mn(2.71 Å)[13] > Fe(2.56 Å)[14] > Zn(2.47 Å)[15] > Co(2.29 Å).[16] The properties of the mixed-metal hybrid hemoglobins can be arranged in a similar sequence on the basis of the relative stability of the T state. The differences in structure between Mn(II) and Zn(II) porphyrins are large, but produce only modest differences in T-state stabilization. This is indicated by the comparable percentages of the slow component in flash photolysis (Table I), which correspond[3] to values of L_2 differing by less than tenfold. The T_2–R_2 equilibrium properties of the relevant diliganded HbA intermediates are undoubtedly bracketed narrowly by those of the (Mn, Fe) and (Zn, Fe) hybrids.

The second type of conclusion concerns the relation between T-state stability and prosthetic group stereochemistry. Our measurements show that the replacement of Fe by Co in two

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chains destabilizes the T state so effectively that unliganded (Co, Fe) tetramers are not fully T_0 in the absence of IHP and that the T_2 state of the doubly liganded (Co, Fe) tetramers is strongly destabilized even in the presence of IHP. This indicates that the values of L_0 ($\approx 10^5$ in HbA[2]) and of L_2 have each been reduced by orders of magnitude through Co replacement in only two chains. Thus, although the structures of the Zn(II) and Co(II) porphyrins are more alike[15,16] than, e.g., the Mn(II) and Zn(II) porphyrins,[13,15] the incremental stereochemical differences between the Co(II) and Zn(II) complexes clearly lead to a hemoglobin with quite different properties from those of HbA. This observation explains the anomalous features in equilibrium measurements of O_2 binding to (Co, Fe) hybrids and the reduced cooperativity of O_2 binding to coboglobin.[17]

These experiments show that mixed-metal hybrid hemoglobins can be used to measure the allosteric properties of and the extent of chain inequivalence within the unliganded and diliganded hemoglobin tetramer; analogous measurements with O_2 as ligand are underway.[3] The use of a series of metals further confirms that the hemoglobin allosteric mechanism does not rely on the details of ferroheme structure in a simple fashion;[18] the dependence appears monotonic, although probably not linear.[19] On the other hand, the results for all three replacement metals show that the CO binding rates of an Fe chain within the Hb molecule are not influenced by the metal occupying the complementary chain.

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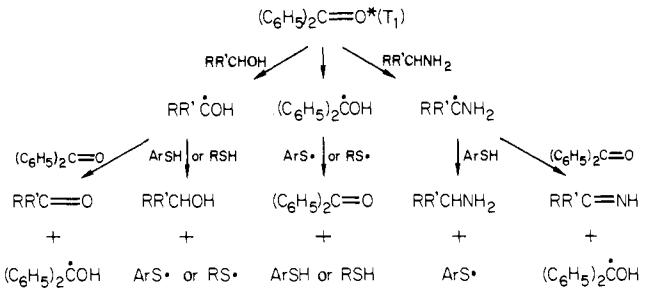
Catalysis by Aliphatic Thiol of Photoreduction of Benzophenone by Primary and Secondary Amines

Sir:

Quantum yields for photoreduction of benzophenone by amines may be substantially increased by aliphatic thiols, and decreased by aromatic thiols,[1] while quantum yields for photoreduction by alcohols are decreased by both aromatic and aliphatic thiols.[2] The decreases have been shown, by deuterium labeling and thiol–disulfide equilibration, to occur by hydrogen-transfer reactions which convert radical intermediates to starting materials (Scheme I).[1,2]

Reduction by amines appears to proceed via initial charge transfer. It was proposed that this would be followed either by proton transfer (k_h) or by regeneration of starting materials (k_e) (Scheme II).[3,4] On this basis, the quantum yield for photoreduction of ketone would depend on the relative importance of the

Scheme I



Scheme II

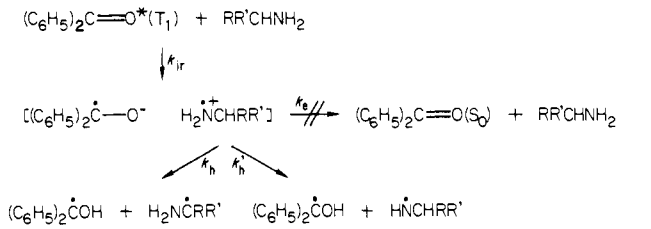


Table I. Photoreduction of 0.06 M Benzophenone by Amines in Benzene. Effect of 1-Pentanethiol and *tert*-Butylamine (TBA)

expt no	reductant compd	M	$(CH_3)_3CNH_2$ trip,[a] M	%	$1\text{-}C_5H_{11}SH$ trip,[b] M	%	φ
1			4.1	100			~0.06
2					0.1–1.0		~0.03
3	$C_2H_5CH(NH_2)CH_3$	0.10					0.79
4	$C_2H_5CH(NH_2)CH_3$	0.10			0.023	1.0	1.20
5	$C_2H_5CH(NH_2)CH_3$	0.10	4.1	92			~0.06
6	$C_2H_5CH(NH_2)CH_3$	0.10	4.1	92	0.020	0.8	1.04
7	$(i\text{-}C_3H_7)_2NH$	0.06					0.56
8	$(i\text{-}C_3H_7)_2NH$	0.06			0.10	1.1	1.29
9	$(i\text{-}C_3H_7)_2NH$	0.06	5.5	78			0.22
10	$(i\text{-}C_3H_7)_2NH$	0.06	5.5	78	0.10	0.2	1.07
11	$(i\text{-}C_3H_7)_2NC_2H_5$	0.06					1.11
12	$(i\text{-}C_3H_7)_2NC_2H_5$	0.06			0.09	0.7	1.02
13	$(i\text{-}C_3H_7)_2NC_2H_5$	0.06	5.5	70			0.36
14	$(i\text{-}C_3H_7)_2NC_2H_5$	0.06	5.5	70	0.02	0.04	0.64

[a] Percent of reacting triplet trapped by TBA. [b] Percent of reacting triplet trapped by the thiol.

k_h and k_e processes, and it was suggested that aliphatic thiol increased quantum yield by catalyzing hydrogen transfer in the charge-transfer complex.[1] However, it has recently been found that reactions of benzophenone with common aliphatic amines, including *tert*-butylamine (TBA) and triethylamine, which have only –NH and α-CH, respectively, form benzophenone ketyl radical with quantum yield $\varphi = 0.9$–1.0; thus, the quenching process (k_e) does not occur significantly.[5] Loss of quantum efficiency must be due to subsequent reactions of the initially formed radicals, and the accelerating effect of aliphatic thiol, like the retarding effect, would involve reactions with these radicals. This has been borne out in studies with TBA (Table I).

Irradiations were carried out, as described previously,[1] on a rotating wheel, along with a secondary actinometer, 0.06 M benzophenone, and 1.2 M 2-aminobutane in benzene, $\varphi = 1.17$. The fraction of triplet reacting with each component was calculated from the rate constants (k_{ir}) and the concentrations. Values of k_{ir}, from phosphorescence quenching,[6] were TBA, 7.0×10^7; 2-aminobutane, 2.5×10^8; and 1-pentanethiol, 1.3×10^7 M^{-1} s^{-1}. The values for diisopropylamine, 1.9×10^9 M^{-1} s^{-1}, and diisopropylethylamine, 2.9×10^9 M^{-1} s^{-1}, were obtained by quenching

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