Kinetics and Equilibria of Soluble Guanylate Cyclase Ligation by CO: Effect of YC-1

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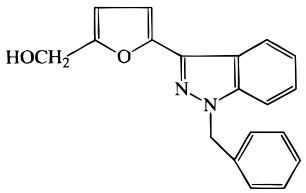
ABSTRACT: Previous work has proved that the enzyme-soluble guanylate cyclase, GC, is activated several 100-fold by the combination of carbon monoxide plus a benzylindazole derivative called YC-1. That is about the same as activation by nitric oxide, which has a well-established role both in vivo and in vitro. This report addresses several spectroscopic, equilibrium, and kinetic effects wrought by YC-1 on carboxyl guanylate cyclase, including the following: a shift in the Soret absorption band by 4 nm to shorter wavelength; an increase in CO affinity by an order of magnitude; a dramatic change in the kinetics of CO association. After photolytic dissociation of CO, the majority, but not all, of bimolecular ligand recombination occurs with a time constant about 1000-fold faster than in the absence of YC-1, while a smaller fraction recombines almost, but not quite, the same as usual. This is reminiscent of the kinetics of NO association with GC, which also shows two prominent phases. The results just listed pertain in the presence of GTP/cGMP, which would be present during enzyme catalysis. Qualitatively similar, but smaller, effects occur in the absence of GTP/cGMP. Measurements are reported to characterize other changes in buffer conditions. The results are consistent with a mechanistic model that attributes a crucial role to the proximal bond that connects the heme iron to a histidine side chain in GC but also requires protein control of the distal environment.

Soluble guanylate cyclase, GC,[1] is implicated in a variety of physiological functions: sensory perception, memory, regulation of vascular tension, and others (*1*−*5*). GC is a heterodimeric heme protein that catalyzes the formation of cGMP from GTP. A major part of the regulation of GC is due to activation by nitric oxide (*6*, *7*). It was surprising a decade ago to find that NO could be a physiological messenger. Now, the question is arising whether CO may also have a regulatory role (*8*) and whether GC may be involved again. Although NO can enhance enzyme activity in GC by a factor of 200−400, CO normally has only a 4-fold effect (*9*, *10*). Surprisingly, the combination of a benzylindazole derivative, code-named YC-1, with CO is synergistic and stimulates in vitro enzyme activity more than 200-fold, comparable to activation by NO (*11*−*13*). This could be pertinent to physiologic effects of CO.



In GC, affinity for both NO and CO is much lower than in myoglobin or hemoglobin; affinity for O_2 is so low that O_2 does not bind noticeably in ambient air. Low ligand affinity in GC is due largely to rapid dissociation. Dissociation of CO takes much less than 1 s (*14*, *15*). Although NO can require a few minutes (*16*), the rate is markedly enhanced by GTP (*17*) and, to a lesser extent, by other factors (*18*). Modest affinity combined with rapid association and dissociation rates is understandable if NO, and possibly CO, is to regulate GC activity. In Mb, NO can take many hours to dissociate spontaneously; even CO is relatively sluggish.

In this report, we explore how CO binding kinetics and equilibria are influenced by simultaneous binding of YC-1. One hypothesis that was proposed previously (*12*) is that the major effect of YC-1 is to increase CO affinity. This was plausible for two reasons: First, CO by itself activates GC weakly, but its affinity is quite low and has scope for a large increase. Second, the fact that CO dissociation is so fast in GC suggests that affinity may raised simply by slowing the dissociation rate constant. Here we test those conjectures. We find that affinity does increase but not enough to account for all the increase in activity, and the increase is not due primarily to slowing the dissociation rate.

It was shown some time ago (*19*) and recently confirmed (*20*) that the heme iron in GC makes a coordinate covalent bond (termed the proximal bond) to a particular histidine,

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[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; CTAB, cetyltrimethylammonium bromide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediamminetetraacetate; GC, soluble guanylate cyclase; GSH, reduced glutathione; GTP, guanosine 5′-triphosphate; Hb, hemoglobin; HSA, human serum albumin; Mb, myoglobin; TEA, triethanolamine; YC-1, [3-(5′-(hydroxymethyl)-2′-furyl)-1-benzylindazole.

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β_1H105. We recently (*21*) summarized our current view of the chemical basis for enzyme activation in GC. Our model owes a great debt to early ideas of Ignarro, Böhme, Schultz, Murad, and Garber as well as recent findings of Marletta, Burstyn, Kitagawa, their associates, and others as well. We put emphasis on the proximal bond and postulate a delicate interplay between 5- and 6-coordination at the heme iron. This model faces a serious challenge from a recent study (*13*) that concluded that addition of YC-1 to GC−CO causes no change in the optical spectrum, the CO affinity, or the CO association kinetics. If those findings are correct, our model and others like it should be cast aside. We report here that behavior of CO in the presence of YC-1 does affect spectra, affinity, and kinetics, and the model remains viable. Partial, preliminary results were reported earlier (*22*).

METHODS

Soluble guanylate cyclase from bovine lung was prepared and characterized as described previously (*6*). YC-1 was obtained from Alexis Biochemicals, San Diego, CA. Results were obtained for 5 different buffers: (A) 50 mM TEA, 3 mM $MgCl_2$, pH 7.4, 3 mM DTT, 0.5 mg/mL BSA, 1 mM cGMP, 0.5 mM GTP; (B) same as (A) without GTP and cGMP; (C) 25 mM TEA, 0.5 mM EDTA, 1 mM GSH; (D) Same as (C) with 50% glycerol; (E) 25 mM TEA, 5 mM DTT, 50 mM NaCl, pH 7.4.

To prepare solutions A−C, buffer was degassed in the sample cuvette by bubbling with argon for more than 1 h, and then equilibrated with the desired CO concentrations by bubbling for 10 min. Finally, degassed protein was added at a dilution of 1:50. Since the protein stock has 50% glycerol, the samples contained 1% glycerol. Premixed CO in argon at certified concentrations was obtained from Matheson.

Kinetic measurements were carried out using laser flash photolysis as described previously (*23*, *24*). Conditions were the following: $\lambda_{ex} = 555$ nm; fluence = 20−30 mJ cm^{-2}; photon absorption at $\lambda_{ex} = 3\%$ for CO complex and 10% for NO complex. The number of laser "shots" averaged for each datum ranged from 20 to 200. Worst case signal-to-noise was S/N = $\Delta A(t = 0)/\delta\Delta A_{rms}$ = 0.006/0.000 05 = 120 for CO complex and 0.0003/0.000 02 = 15 for the NO complex for bimolecular association and about 4 times less for nanosecond geminate recombination measurements. Sampling times were varied to ensure that no significant transients escaped detection. For CO, time courses for bleaching or transient absorption were monitored at 423−424 nm or at 435 nm, respectively. For NO, the wavelengths used were 408−412 and 435 nm. Occasionally measurements were made throughout the range 385−450 nm to verify that no unexpected transients were present. We searched for and characterized clean isosbestic points. The temperature was 23 ± 0.5 °C.

RESULTS

Spectra and Equilibria. Any change in the ligation state of the heme iron should manifest itself in some change in the UV−vis absorption spectrum. Figure 1 shows that this is the case. It also shows a significant effect of GTP. Adding YC-1 to a solution of GC−CO causes a shift of 4 nm in the Soret absorption peak in the presence of GTP but only half that in the absence of GTP.

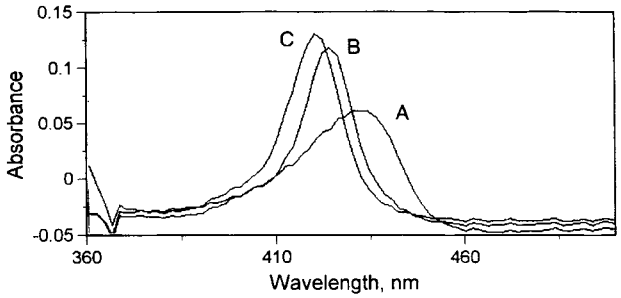


FIGURE 1: Soret spectra for (A) GC (λ_{max} = 432 nm), (B) GC−CO (λ_{max} = 424 nm), and (C) YC-1-GC−CO (λ_{max} = 420 nm), recorded with GTP present buffer A: [CO] = 1 atm; [GC] = 1 μM; [YC-1] = 130 μM. Omitting GTP has no effect on (A) or (B) but produces a smaller shift for (C) (λ_{max} = 422 nm).

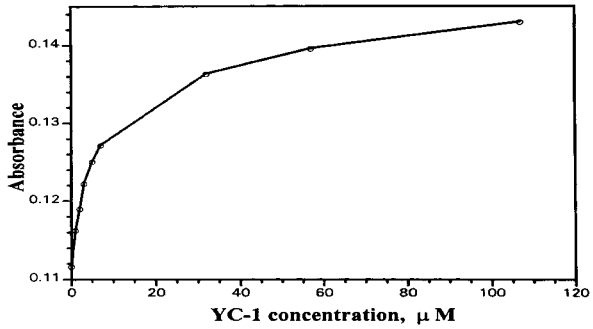


FIGURE 2: Titration of GC−CO with YC-1, in the presence of GTP in buffer A: [CO] = 1 atm; [GC] = 1.2 μM; ordinate is $A_{420} - A_{460}$.

The spectral shift allows some estimation of equilibrium binding constants by titration. Figure 2 shows incremental additions of YC-1 to GC−CO. The half-saturation point suggests that, in the presence of GTP, $K_a = K_{CO}^{YC-1} \approx 125$ mM^{-1}. In this notation, the component present in large excess is the subscript, while the component varied in the titration appears as the superscript.

Formation of YC-1−GC−CO should be feasible in either order. Figure 3 illustrates the effect of adding CO to YC−1-GC. In the presence of GTP, $K_{YC-1}^{CO} \approx 200$ mM overall, but there are clearly two phases with apparent $K_{strong} \approx 250$ mM^{-1} and $K_{weak} \approx 10$ mM^{-1}. The interpretation of the latter number is model dependent, if two forms of YC-1−GC are in equilibrium. There may also be more than two spectra involved, but they cannot be resolved. It is essential to consider kinetic data. The question whether YC-1 affects CO binding is addressed directly in Figure 3. Without YC-1, $K^{CO} \approx 22$ mM. The effect of YC-1 is to increase the affinity of GC for CO by 1 order of magnitude and to impose a two-phase behavior on the titration.

The question arises whether YC-1 affects other heme protein−CO systems. For horse heart Mb−CO, the Soret band is at λ_{max} = 424 nm in the absence of YC-1 and does not shift at all when YC-1 is added at the same concentrations used for GC. In HSA−CO, the heme is incorporated solely by hydrophobic interactions, with no proximal linkage. In the absence of YC-1, λ_{max} = 417 nm. Addition of YC-1 causes a shift to 420 nm. Note that this shift for YC-1−HSA−heme−CO is in the opposite direction from that in the guanylate cyclase ternary complex, but the shifted Soret band position is at the same λ_{max} as in YC-1−GC−CO. Could it be that the same species, perhaps even YC-1−Fe−

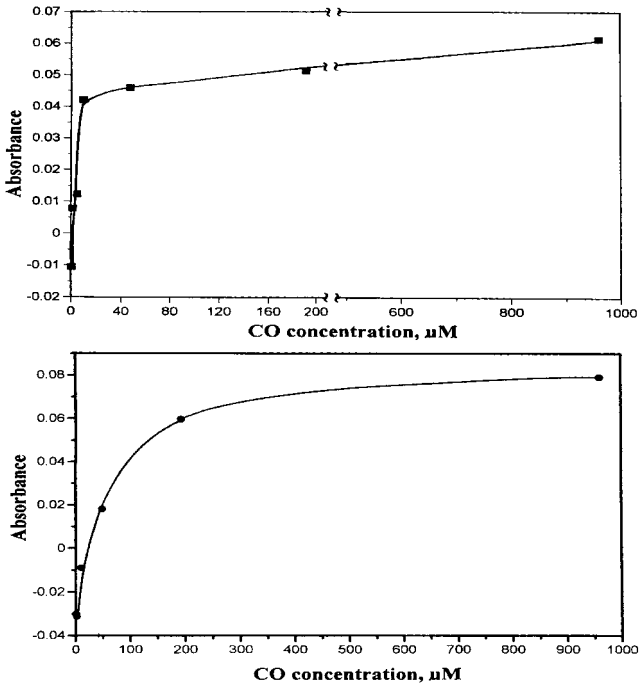


FIGURE 3: Top: Titration of YC-1−GC with CO, in the presence of GTP in buffer A: [GC] = 1 μM; [YC-1] = 200 μM; ordinate is $A_{420} - A_{432}$. Bottom: Titration of GC with CO in the presence of GTP, buffer A, without YC-1: [GC] = 1 μM; ordinate is $A_{420} - A_{432}$.

CO, might be present in both GC and HSA but not in Mb, where a stronger proximal Fe−His bond is better able to maintain its integrity?

Kinetics. In the first approximation, the kinetic analysis characterizes a bimolecular association mechanism:

$$\mathrm{GC} + \mathrm{L} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{GC{-}L} \qquad (1)$$

If exponential fits apply for a range of concentrations of the free ligand [L], thereby confirming the mechanism, the observed combination rate, k_{obs}, is interpreted as

$$k_{obs} = k_a[\mathrm{L}] + k_d \qquad (2)$$

In the absence of YC-1, this simple model works well. In the presence of YC-1, however, the situation is more complicated. There are two phases to the bimolecular association process, and in addition, a much faster, concentration-independent phase appears that is ascribed to geminate recombination of CO with iron before it can escape from the protein.

Reaction of GC with CO in the Absence of YC-1. Typical data are shown in Figure 4 for buffer A, that is, with GTP present. There was an excellent isosbestic point at 429.3 ± 0.5 nm. Similar measurements were made for solutions equilibrated with CO at partial pressures ranging from 0.0050 to 1.00 atm. The k_{obs} were plotted vs [CO] according to eq 2. There were 27 data points, which include all the measurements attempted; no "practice" data were discarded. Assuming a solubility for CO of 940 μM atm $^{-1}$ at 23 °C, the results obtained from the slope and intercept were: In the presence of GTP and cGMP, $k_a = 81 \pm 2$ mM^{-1} s^{-1} and $k_d = 10.3 \pm 0.6$ s^{-1}. The equilibrium constant for association is $K_a = k_a/k_d = 7.86 \pm 0.50$ mM^{-1}. This is a rather low affinity for CO binding and implies that the heme iron is half saturated at about 0.12 atm of CO. Compared with Mb (sperm whale or horse heart), the association rate is not quite 10 times smaller; the dissociation rate is about 500 times faster and affinity is roughly 4000 times lower.

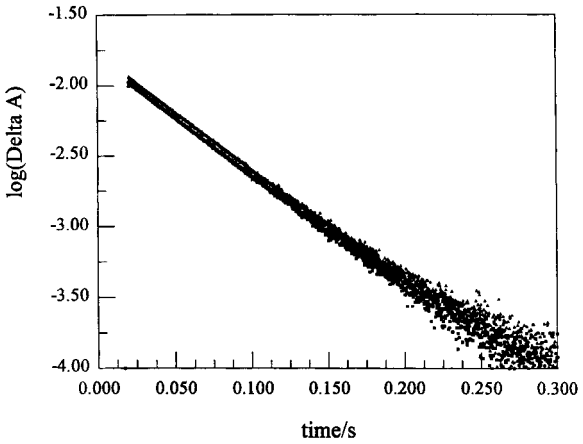


FIGURE 4: Base-10 semilog plot of transient absorption changes following flash photolysis of GC−CO in buffer A without YC-1, in the presence of 93 μM [CO]. Upper trace is the absolute value of a bleach at 423 nm; the lower curve is a transient absorption at 435 nm for the same sample. Single exponentials fit well, but not perfectly, over more than 6 half-lives.

Recombination of CO with GC was characterized for other buffers with the results shown in Table 1. Buffer B is the same as buffer A, except that it lacks GTP and cGMP (and $MgCl_2$). The kinetics observed show little difference between buffers A and B. For both buffers A and B, recombination could be fit well, but not perfectly, by a single exponential. Careful measurements revealed a small amplitude component with an observed rate 2−6 times slower. This component was hardly noticeable in buffer A but contributed as much as 10−20% amplitude in buffer B. We attribute this to heterogeneity in the protein, heterogeneity that is reduced when GTP is present. We do not see such behavior in Mb. It could be that the complex purification for GC inevitably results in a small fraction of slightly damaged protein, but it is also plausible that the catalysis carried out by the enzyme requires it to exhibit some variability in conformation that affects rates.

Buffers C−E are included in order to make contact with previously published results. Buffer D is the one we used previously to demonstrate that CO dissociation from GC is extraordinarily fast (*14*). It is clear that buffer D is measurably different from buffer A. At that time, the available stock protein solution in 50% glycerol was so dilute that it had to be used in that condition, but there were other small differences as well. To separate the effect of glycerol from other factors, we prepared buffer C, which matched the old buffer D except that it had less than 1% glycerol. We conclude from buffers B and C that variations in buffer composition can affect rates and from buffers C and D that a large admixture of glycerol also has an effect. Although measurable, all this variation among buffers is negligible compared with the difference between GC and the globins. Buffer E is that used in a study by Stone and Marletta (*15*). They used mixing rather than flash photolysis. It is reassuring that the results are similar. Most of the small variation

Table 1: Kinetics and Equilibria for CO Binding to Soluble GC in the Absence of YC-1

protein, ligand, cofactor	buffer	T, °C	nanosecond geminate, %	k_{on}, mM^{-1} s^{-1}	k_{off}, s^{-1}	K_a, mM^{-1}	ref
GC, CO	A	23	<5	81 ± 2	10.3 ± 0.6	7.86 ± 0.50	this work
GC, CO	B	23	<5%	82 ± 2	11.4 ± 1	7.19 ± 0.67	this work
GC, CO	C	23	<5%	98 ± 2	6.7 ± 1	14.6 ± 2.2	this work
GC, CO	D	23	NM[a]	120 ± 10	28 ± 2	~4	*16*
GC, CO	E	10	NM	35.8 ± 1.5	3.5 ± 0.5	10.2 ± 1.6	*17*
GC, CO	A	23				~22[b]	this work
GC, CO	B	23				~10[b]	this work

[a] No measurement attempted. [b] Estimate from titration.

reflects, no doubt, the different temperatures, but some may be due to slight differences in the buffer.

We searched for possible geminate rebinding of CO before it escaped from the protein, from 50 to 500 ns. Any geminate recombination on the nanosecond time scale is less than 5%.

Reaction of GC with CO in the Presence of YC-1. In the presence of 200 μM YC-1 in buffer A, the association kinetics of CO ligand change dramatically. This 200 μM concentration is the same as used previously for studies of enzyme activity (*11*); it is enough to saturate that activity, and it is also enough to reach a "plateau" in CO ligation kinetics. The overriding effect of adding YC-1 is that, after photolysis, most of the transient absorption changes occur at a rate 1000-fold faster than in the absence of YC-1. The remaining, minor fraction of the transient response occurs on approximately the same time scale as in the absence of YC-1, although with somewhat smaller k_d. Typical time courses were displayed earlier (*22*). Close study of the isosbestic region revealed that the fast component has an isosbestic shifted by about 2 nm toward shorter wavelengths (exactly what is expected when the initial spectrum shifts 4 nm), but the slow phase has little or no shift. The presence of two phases and the fact that the slow phase shows no apparent shift suggests that two populations coexist, consistent with indications from Figure 3.

Since the two phases are so distinct in time scale, they can be analyzed separately to plot the fast process, k'_{obs}, and the slow process, k_{obs}, vs [CO]. The plots are shown in Figure 5. All measurements are plotted. Linear fits prove that both processes involve bimolecular association. Slopes and intercepts yield the rate constants collected in Table 2. There is little to say about the slow process, which is similar to but not quite identical to that observed in the absence of YC-1. Characterizing the fast component raises special problems with regard to k'_d. We used concentrations as low as 0.50% and 0.10% atmosphere of CO to minimize the extrapolation to the intercept. Although not evident in the figure, it seems clear that k'_d is roughly 100-fold greater than for the slow component, k_d, which in turn is already about 500-fold greater than what we are used to for CO in oxygen-binding globins. Fortunately, the fast process is the simpler part of the titration curve to analyze and the K' measured there, together with k'_a, confirms the intercept k'_d.

We investigated the effect of GTP and of YC-1 concentration on the two phases. The second entry in Table 2 shows that in buffer B, which lacks GTP and cGMP (and also $MgCl_2$), the fast process is detectable but reduced in amplitude. It is noteworthy that GTP/cGMP has an effect only in the two cases in which GC is highly activated, namely, either with NO (*17*) or with the combination of YC-1 and CO but not with CO alone. The next entries in Table 2 show that the amount of fast component is proportional to the concentration of YC-1, up to a plateau that has less than total fast phase. Since the YC-1 has to be solubilized in DMSO, we verified that DMSO has no noticeable effect by itself, as shown in fifth row of Table 2.

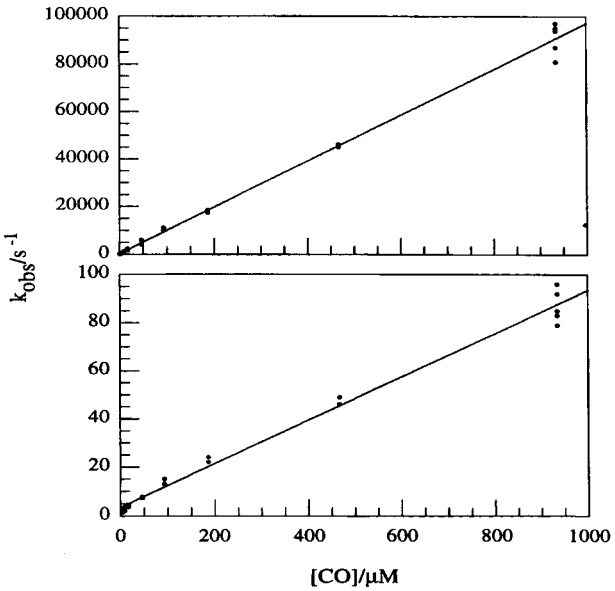


FIGURE 5: Observed fast and slow rate constants versus [CO] for bimolecular recombination following photolysis of YC-1−GC−CO in buffer A.

We searched for nanosecond geminate recombination and found substantial amounts whenever there is a large amplitude of the fast process. As the fast bimolecular component vanishes at low [YC-1], so also does the nanosecond geminate recombination. According to the contemporary model for ligand rebinding in other heme proteins (*25*), geminate recombination should accompany any fast bimolecular association.

The slight heterogeneity seen in the absence of YC-1 is barely detectable in both phases in the presence of YC-1. One concern that might be raised is whether the pseudo-first-order approximation might fail at the lowest [CO], for which both total protein and total CO have concentrations near 1 μM. At the lowest concentration so much ligand remains unbound that the [CO] is in excess and does not change much over the time course of recombination, <20% even if we could photolyze all bound CO and less for the actual photolysis level.

Finally, consider the relative amplitudes of the fast and slow processes as a function of [CO], as shown in Figure 6. Down to 50−100 μM CO, the ratio remains approximately

Table 2: Kinetics and Equilibria of Ligand Binding to Soluble GC in the Presence of YC-1

protein, ligand, cofactor	[YC-1], μM	buffer	ns gem, %	fraction of fast,[a] %	k'_a, $\mu M^{-1} s^{-1}$	k'_d, s^{-1}	k_a, $mM^{-1} s^{-1}$	k_d, s^{-1}	K'_a, mM^{-1}	K_a, mM^{-1}	$K_{overall}$, mM^{-1}
GC, CO, YC-1	200	A	20 ± 5	50−60	97 ± 2	400 ± 200	90 ± 3	3.5 ± 1.2	240 ± 120	26 ± 8	~39
GC, CO, YC-1	200	B	NM	~22	~90	NM	100 ± 10	4 ± 2			
GC, CO, YC-1	115	A	~25	~53	~95	NM	~90	NM			
GC, CO, YC-1	15	A	~10	~23	~92	NM	~90	NM			
GC, CO	0[b]	A	<5	<2			~90	NM			
GC, NO		A	<50	75−85	180	<2000	1200	<10			
GC, CO, YC-1	200	A							~250[c]		

[a] At 20−50% atm [CO]. [b] This entry differs from that in Table 1 in that 1% DMSO is present. [c] Estimate from titration.

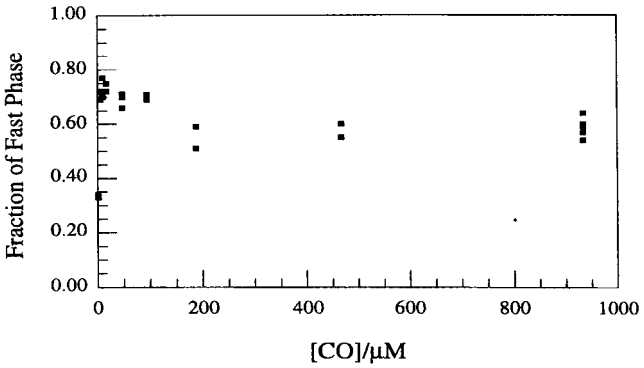


FIGURE 6: Fractional amplitude of the fast component as a function of [CO], as observed in the bimolecular recombination following flash photolysis of YC-1−GC−CO in buffer A.

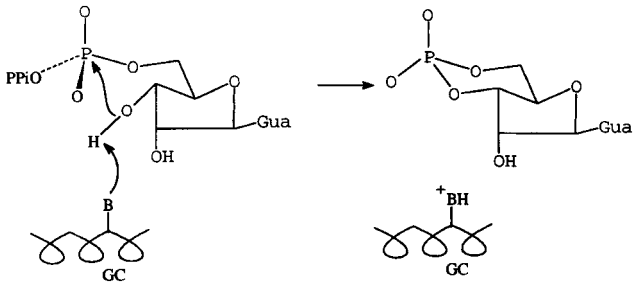
constant. Below that there is an increase in the fraction of fast component. This confirms that the fast process has tighter binding, that is $K'_a > K_a$, so that the fraction of CO bound to the slow reacting form diminishes first. This interpretation is consistent with the titration shown in Figure 3, which reveals tighter binding below about 50 μM [CO]. At the lowest [CO], the fast fraction seems to diminish abruptly. A likely explanation is suggested below.

Reaction of NO with GC. The presence of two very distinct phases for the combination of CO with GC in the presence of YC-1 is reminiscent of NO association kinetics, for which stopped-flow mixing revealed two distinct kinetic phases on much different time scales, as reported by Stone and Marletta (*7*). To exclude the remote possibility that there is some difference between mixing and flash photolysis, we verified that NO recombination with GC after flash photolysis also exhibits two distinct phases. We also proved that this behavior persists in the presence of GTP. Parameters found are summarized in Table 2.

DISCUSSION

That YC-1 can activate platelet guanylate cyclase was reported in 1994 by Ko et al. (*26*). The synergistic effect of YC-1 plus CO was reported in 1996 by Friebe et al. (*11*). Two years later, a more extended study by Friebe and Koesling (*12*) motivated the work reported here. It elaborated on catalytic aspects, deduced that CO affinity was enhanced, and noted no very apparent change in UV spectral properties. At about the same time, Stone and Marletta (*13*) confirmed the catalytic synergism and noted the lack of any change in UV spectra but went further in reporting no change in association kinetics, so far as they could tell by their methods for their sample conditions. They inferred that their results raise a serious question whether bonding at the heme iron is as important for activation as had been thought. Fortunately, the difference between that study and the present results is easily explained. Although studies of catalysis have GTP in their sample preparations, physical measurements, including those in ref *13*, generally omitted that component. Until our report on NO dissociation (*17*), no one imagined that GTP would affect ligand binding. Although all of the observations we report persist in the absence of GTP, they are much more evident with GTP present. This, in combination with the superior time resolution possible with flash photolysis, accounts for the failure of the mixing studies to detect either the small spectral shift or the dramatic change in association kinetics. It was also shown that GTP and cGMP affect the Raman spectrum of GC−NO but not that of GC−CO (*27*). It would be interesting to learn the results of a Raman study of YC-1−GC−CO.

The fact that unusual, complex kinetics for ligand association are found in the two situations in which GC is highly activated is a strong hint that the same mechanism is operative in both cases. Senter et al. (*28*) suggested as early as 1983 that the conversion of GTP to cGMP could be accomplished by base-catalyzed direct displacement:



Binding NO to a heme iron opposite a proximal histidine is expected to weaken the proximal bond. In model hemes, the bond breaks readily at neutral pH, as indicated by a shift in Soret absorption to below 400 nm. In the oxygen-binding globin proteins and some cytochromes, the protein stabilizes the bond so that it does not break at neutral pH, but the bond still breaks at low pH (*29*). In GC, as in the models, the bond apparently breaks, or greatly weakens, even at neutral pH, as evidenced by a spectral shift to 398 nm.

If breaking the proximal bond to β_1H105 is the key to activating GC, there are two options for the Senter mechanism. Breaking (or just weakening) the proximal bond could permit conformational changes that expose a base elsewhere in the protein, or conceivably, the proximal histidine itself might swing into an accessible position and serve as the Senter base. That a proximal imidazole can be released by bonding NO opposite and then catalyze a reaction was

demonstrated conclusively using a model compound (*30*). Far from excluding either possibility, the data reported here are still consistent with both these possibilities.

Unlike the negative trans-effect in heme−NO, binding CO to a heme iron normally leads to a positive trans-effect, which strengthens both the proximal and distal bonds. How then could one explain activation both by NO and by the combination of YC-1 + CO using essentially similar mechanisms? How can one account even for the small enhancement by CO alone? In our view, the protein must apply constraints on the proximal linkage, and also on the distal environment, that render it difficult to maintain both bonds. If that is possible, it is a small additional step to suggest that YC-1 may enhance the constraints.

The first possibility for the Senter mechanism, a generalized conformational change, might be accomplished without actually breaking the proximal bond. Long ago it was observed (*31*−*34*) that when inositol hexaphosphate was added to nitrosyl hemoglobin, a small blue-shift in the Soret spectrum was accompanied by the three-line EPR spectrum characteristic of high-spin iron. The observations were attributed (*35*) to the proximal bond being stretched and weakened. At the same time, IHP treatment shifted the Hb toward the quaternary T structure. Apparently, small changes in bonding at the iron, accompanied by only a small change in the Soret spectrum, are all that is needed to effect significant conformational change. Then YC-1 need only impose sufficient strain to perturb the Soret spectrum and lengthen the proximal linkage. Of course, actually breaking the bond should serve as well, but that might amount to "overkill".

The other mechanism for activation, invoking β_1H105 itself as the Senter base, seems almost too simple to be real, but it is an interesting conjecture to investigate. It would seem to require total cleavage of the proximal bond followed by substantial motion of the histidine. Even this breaking of the bond to β_1H105 can be consistent with retention of a six-coordinate iron in YC-1−GC−CO, as preferred for the positive trans-effect of CO. All that is needed is to replace β_1H105 by another base, either another amino acid side chain or even the YC-1 itself. Such a change from one proximal ligand to another would be expected to cause only a small shift in the Soret absorption feature, as observed. Finally, an enzyme need not retain a particular structure throughout its activity cycle. One could imagine a cycle in which the critical bond is in equilibrium between "made" and "broken." We invoked that interpretation to explain both rapid dissociation for CO and slight activation by CO (*14*, *21*). Existing Raman data make it clear that GC−CO is 6-coordinate, or at least that any equilibrium is strongly biased in that direction (*36*), but cannot exclude the existence of a minor fraction that is different in some way. In this model, adding YC-1 should stabilize the broken proximal bond, and what better way to do that than by substituting a bond to another base, leaving β_1H105 free but keeping the 6-coordinate Fe−CO? This is all speculative, but it seems plausible, if GC destabilizes all 6-coordinate, low-spin forms but CO always maintains a positive trans-effect so far as it can.

The fast dissociation found for both CO and NO is as striking as the two-phase association kinetics. Fast dissociation allows reduced affinity, so that GC is not permanently tied up by bound ligand, while also allowing rapid kinetic response. We proposed that an equilibrium between five- and six-coordinate forms of heme ligation could account for fast ligand release of both CO (*14*) and NO (*16*). Interestingly, the state responsible for fast dissociation should be opposite for the two ligands: NO should be released very quickly from the six-coordinate form (*16*), while CO should be released more rapidly from the five-coordinate species (*37*). This may seem surprising, but proteins do surprising things and we know of no alternative hypothesis.

The presence of two kinetic phases can be understood with the following kinetic model: Prior to photolysis, some

$$\begin{array}{ccc} \mathrm{GC'} + \mathrm{CO} & \underset{k'_d}{\overset{k'_a}{\rightleftharpoons}} & \mathrm{GC'\text{-}CO} \\ k_1 \uparrow\downarrow k_2 & & k_3 \uparrow\downarrow k_4 \\ \mathrm{GC} + \mathrm{CO} & \underset{k_d}{\overset{k_a}{\rightleftharpoons}} & \mathrm{GC\text{-}CO} \end{array} \qquad (3)$$

equilibrium exists among the four species (and possibly other minor constituents). Photolysis perturbs the distribution by dissociating CO from the species on the right-hand side and increasing the concentrations of those on the left. Recombination occurs in parallel and independently for all but the longest times, with GC′ reacting faster. This requires that GC′ and GC remain distinct throughout most of the recombination, which requires that k_1 and k_2 be sufficiently slow. With these assumptions, k'_a, k'_d, k_a, and k_d in eq 3 can be measured separately and are the constants in Table 2. (If equilibrium were achieved between GC′ and GC prior to recombination, only one relaxation would be observed.) The ratio k_3/k_4 can be estimated from the ratio of fast to slow amplitude at large [CO], when all hemes are liganded prior to photolysis. According to Figure 6 this is about 0.6, assuming that photolysis is equally efficient for both forms. At fairly low [CO], the form with higher affinity will have higher ligation. Since $K'_a > K_a$, the fraction of fast phase should increase at low [CO], as is the case in Figure 6. At the very lowest [CO], recombination is very slow and GC′ can convert to slower-reacting GC before recombination is completed. Then the fraction of slow recombination will increase. (This is quite similar to the R → T transition in Hb.) That also is seen in Figure 6. The characteristic time at which this happens implies $k_2 \approx 1\ s^{-1}$.

A strong argument for the existence of a parallel reaction by two species is the presence of two isosbestic points and the ability to find wavelengths at which only one process is observable, with the same characteristic rate as always. The apparent lack of a rise time at any wavelength also argues against a serial mechanism, in which all deliganded GC is identical at any point in time but evolves from a fast-reacting to a slow-reacting form. The serial model is unlikely for a second reason. Such a change should terminate the fast process after a fixed time, shifting amplitude toward the slow phase. The fact that the fast process is proportional to [CO] and remains at almost constant amplitude over a wide range of [CO] favors a parallel model. At the very lowest [CO], however, a sequential process does set in, as discussed above.

Not all of the constants in mechanism (3) can be independent, because it expresses equilibrium around a com-

plete cycle. Consequently, we know

$$\frac{k_2}{k_1} = \frac{k_4 k'_a k_d}{k_3 k'_d k_a} = \frac{k_4 K'_a}{k_3 K_a} \quad (4)$$

Since k_3/k_4 is about 0.6, we can deduce that $k_2/k_1 \approx 15$, which means that the equilibrium between unliganded GC and GC′ strongly favors GC, with $k_2 > k_1$. From the rough estimate $k_2 \approx 1\ s^{-1}$, we can then deduce that $k_1 \approx 0.07\ s^{-1}$. For k_3 and k_4, only the ratio is available. Since $k_3/k_4 \approx 9k_1/k_2$, binding CO has about a 10-fold effect in biasing equilibrium away from GC toward the faster reacting GC′, which is presumably the catalytically more active form. Note that the slower form GC already has an affinity for CO that is about 3−4 times greater than in the absence of YC-1, while the fast reacting GC′ has another 10-fold increase in affinity.

The question arises for the complex mechanism (3), what is the overall equilibrium for adding CO to the deliganded forms on the left to produce the liganded forms on the right? This can be written from the kinetic rate constants, and we can eliminate GC′ and GC′−CO from the expression to reach:

$$K_{overall} = \frac{\left[\mathrm{GC{-}CO}\left(1 + \frac{k_3}{k_4}\right)\right]}{\left[\mathrm{GC}\left(1 + \frac{k_1}{k_2}\right)\right][\mathrm{CO}]} = K_a \frac{1 + k_3/k_4}{1 + k_1/k_2} \quad (5)$$

We expect $K_{overall}$ must lie somewhere between K_a and K'_a, that is, between 26 and 240. In fact, $K_{overall} = 1.5K_a = 39$.

We may, finally compare this calculated $K_{overall}$ with the equilibrium constant in the absence of YC-1, as recorded in Table 1. The overall, effective enhancement in CO affinity due to the presence of YC-1 must be, by this method, very close to 5.

What distinguishes GC′ from GC? It could be that GC′ has YC-1 bound but GC does not. In favor of this is the fact that GC behaves somewhat like the protein without YC-1. Arguing against that simple hypothesis are two observations: First, the slow phase, involving GC, has behavior that is not, even considering uncertainties, identical to what is seen in the absence of YC-1; second, the amplitude of the fast bimolecular phase plateaus at 60% of the total even in the presence of a large excess in YC-1. It seems more likely that YC-1 is binding to all protein, but there are then two forms of protein in equilibrium, one fast reacting and one slow reacting.

It was originally a great surprise when GC was reported to show association kinetics with NO that displayed two phases differing in characteristic time by a factor of 100 or more (*7*). That was such a dramatic result that we confirmed the general features. We do find some differences, due mainly to the better time resolution available by using flash photolysis. We plan to discuss NO kinetics in another place. We only report enough data here to be sure that it is valid to point to a similarity in association kinetics between YC-1−GC−CO and GC−NO, and there are no unexpected differences between mixing and flash photolysis nor any affect of GTP.

CONCLUSION

The kinetics of CO ligation with GC are markedly changed by the presence of YC-1. A new, faster phase appears in all cases; it dominates in the presence of GTP. In that fast phase, association and dissociation increase by factors of roughly 1000 and 100, respectively. Ligand affinity also increases by about 1 order of magnitude. At the same time, the Soret band shifts from 424 to 420 nm. A specific kinetic model was developed that is consistent with all the data. Other models, of course, might also fit the data. These changes suggest to us that, in the presence of YC-1, the proximal bond to β_1H105 is weakened or replaced by a bond to a different base. Both alternatives are consistent with the hypothesis that the active state is similar in some essentials to that in GC−NO, and they are consistent with the traditional idea that the state of the proximal bond is one key aspect of GC activation.

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