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Soluble Guanylate Cyclase: Effect of YC-1 on Ligation Kinetics with Carbon Monoxide

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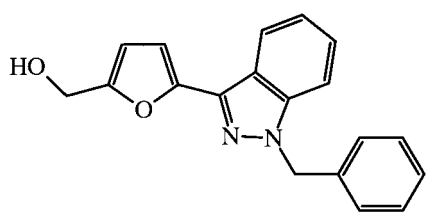
Recently it has been reported that in the presence of YC-1, a benzyl indazole derivative, carbon monoxide activates soluble guanylate cyclase, GC, to about the same extent as its best known activator, nitric oxide. Kinetic studies utilizing flash photolysis of GC complexed with CO in the presence and absence of YC-1 show, in contrast to another recent report of a mixing experiment, that YC-1 has a profound effect on bimolecular association kinetics and a smaller, but significant, effect on ligand affinity. Most prominent is the appearance of a major, new phase in the bimolecular recombination kinetics in the presence of 200 μM YC-1: This major fraction rebinds CO ~1000-fold more rapidly than in the absence of YC-1. Another portion, considerably less than half, exhibits kinetics that are almost exactly the same as in the absence of YC-1. It is now clear that both YC-1 and CO have a strong synergistic effect on enzyme activity and also a dramatic effect on ligand binding behavior. It is, therefore, a reasonable inference that ligand binding at the heme iron atom is intimately connected with enzyme activation, a hypothesis that would have been difficult to maintain if the earlier report, that YC-1 has no effect on CO binding, were correct. Possible reasons for the discrepancy between the two measurements are suggested.

The intra- and intercellular messenger molecule nitric oxide, whose importance is now well recognized, exercises many of its physiological effects on sensory perception, memory, vascular tone, etc. (1–3) by stimulating the heme protein soluble guanylate cyclase, GC (4, 5). Binding NO to the heme iron atom in GC enhances enzyme activity more than 200-fold (6, 7). Binding NO is also accompanied by a shift in the Soret absorption band from 420 to 398 nm (8), which is convincing evidence that the bond between the iron and a histidine residue in the protein is broken when NO binds. That bond is termed the proximal bond. It was shown that the residue involved is β_1H105 (9); an assignment that that was confirmed recently (10).

Based just on those observations, one hypothesis that may be entertained for the mechanism of GC activation is that breaking the proximal bond facilitates some change in the overall three-dimensional structure of GC that speeds catalysis. Well before the full importance of NO was recognized, Senter *et al.* made a convincing case that GC catalysis depends essentially upon proton extraction by a base (11). Changing the protein structure could expose a basic side chain. In fact, it could even be that the proximal base itself is the Senter base. Support for this option was the demonstration that binding NO can break the proximal bond in a chelated model heme system and activate that model heme to participate in a catalytic reaction (12).

There are, therefore, three possible hypotheses to be distinguished: (a) It is entirely fortuitous that breaking the proximal bond in GC-NO happens to correlate with enhanced catalytic activity; in principle species with or without proximal bonds may have either high or low activity. (b) Breaking the proximal bond is important, but only through some effect on the structure of GC. (c) The proximal base itself is involved in catalysis and must be freed from the iron before it can accept a proton and catalysis can occur. For the purpose of deciding among these alternatives, YC-1 offers a compelling opportunity. Results communicated here suggest that hypothesis (a) is rather unlikely. They are perfectly consistent with hypothesis (c), but hypothesis (b) cannot be ruled out.



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Soluble guanylate cyclase binds CO and is slightly stimulated thereby (typically 4-fold) (13, 14). That GC is also slightly stimulated by YC-1 was first proved by Ko *et al.* (15). The enhancement of activity by YC-1 alone was quantified as 10-fold in one report (16) and 5-fold in another (17). When present together, however, CO + YC-1 are synergistic and activate GC to about the same level as does NO (>200-fold) (16, 17). If the proximal bond has some direct connection to enzyme activity, there should be some effect on spectral and thermodynamic properties of the heme group even with YC-1 alone bound or with CO alone; but such effects might be too small to be seen, since either alone induces only a slight enhancement of enzyme activity. When both YC-1 and CO bind together, however, one should, if the proximal base is important, expect quite apparent effects on kinetic and/or spectroscopic behavior at the heme, well beyond those induced by either alone. For this reason, a recent report by Stone and Marletta (17), that there are no such effects, is of extreme importance. Those authors reported that, ". . . YC-1 has no effect on the on rate (k_{on}) or off-rate (k_{off}) for CO binding to the heme. . . . YC-1 CO forms a 6-coordinate complex . . ." and concluded, quite correctly, "On the basis of these results, the requirement for cleavage of the Fe-His bond to achieve full activation of sGC is called into question." Experimental results reported here, obtained under somewhat different conditions, however, do not agree that there is no effect of YC-1 on ligation kinetics.

MATERIALS AND METHODS

Protein solutions, except for added YC-1, were the same as used in a prior kinetic study of NO dissociation (18), which in turn were chosen to mimic those used for investigations of enzyme activity (16). The GC is from bovine lung and used at about 1 μM concentration, in solution with 50 mM triethanolamine, 3 mM $MgCl_2$, 0.5 mM GTP (guanosine 5′-triphosphate), 1.0 mM cGMP (guanosine 3′,5′-cyclic monophosphate), 3 mM dithiothreitol, 0.5 mM bovine serum albumin, 200 μM YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole), 1.5% V/V dimethyl sulfoxide, and <1% glycerol. The flash photolysis apparatus was also as described previously (19, 20). Key parameters include: 1.00-cm pathlength for the probe beam; 2 mJ excitation energy in 4 ns over a cross section of about 6 mm^2, colinear with the probe; 100 to 200 laser shots summed for each curve; temperature, 23 ± 0.4°C. Gas mixtures of CO diluted with argon were premixed by the supplier and solutions were equilibrated carefully for about 1 hour before measurement commenced.

RESULTS AND DISCUSSION

Kinetic traces for the association of CO with GC following laser flash photolysis of solutions at two different CO concentrations are shown in Fig. 1. From the data in Fig. 1 along with data at other [CO], the results in Table 1 were derived, assuming $k_{obs} = k_a[CO] + k_d$. In such perturbation kinetic experiments, it is customary to plot the absorbance change, ΔA, vs time. The

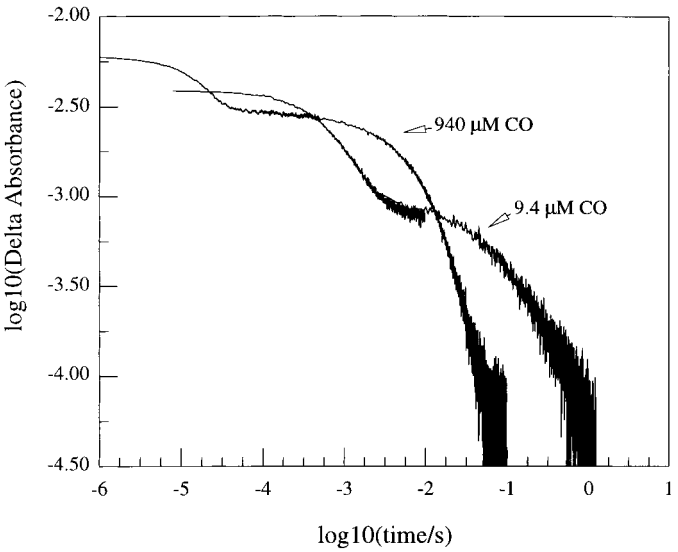

FIG. 1. The common log of the change in absorbance at 436 nm is plotted vs the common log of time after laser photolysis of solutions of GC-CO-(YC-1), for two different CO concentrations. [YC-1] = 200 μM; [GC] = 1 μM. The smaller total amplitude for the lower concentration arises because GC-heme is only partially at that [CO]. For each concentration, data were collected on two different settings of the time base of the digitizer and spliced to make the figure.

data are displayed as log(ΔA) vs log(time) in order to facilitate easy comparison with the data of Stone and Marletta (17). Unlike that report, under our conditions, there is a very dramatic effect due to YC-1. In a log-log plot of this type, a single exponential decay process appears as a "waterfall-shaped" plunge from one plateau to another, lower plateau. Ultimate return to the preflash equilibrium, however, appears at minus "infinity." The two curves show data for 1.0 atm of [CO] and for 0.010 atm of [CO]. At both these widely different CO concentrations, the association kinetics display two very distinct phases, each more or less a single exponential. Both phases increase in rate proportional to [CO], so both are second-order combination processes. For the slow phase in the presence of YC-1, Table 1 reports a modest decrease in the dissociation rate constant, k_d, and a barely significant increase in the association rate constant, k_a, relative to values obtained for the same solution in the absence of YC-1. These values are all generally similar to what was observed previously in the absence of YC-1, as reported by ourselves (20) and others (21). They are also similar to what was observed by Stone and Marletta (17) as the entire combination reaction in the presence of YC-1, when allowance is made for different temperatures and solution compositions. In our experiments, however, the slow process accounts for considerably less than half of the total recombination after photolysis. The effect of YC-1 turns out not be a change in a single CO binding process, but rather the appearance of a new kinetic process, three orders of magnitude faster, that

TABLE 1

Kinetics for CO Binding to Soluble Guanylate Cyclase with and without YC-1

Protein, ligand	[YC-1] (μM)	k'_a ($\mu M^{-1}\ s^{-1}$)	k'_d (s^{-1})	k_a ($\mu M^{-1}\ s^{-1}$)	k_d (s^{-1})	k'_a/k'_d (mM^{-1})	k_a/k_d (mM^{-1})
GC, CO	200	97 ± 2	400 ± 200	90 ± 3	3.5 ± 1.2	240 ± 120	26 ± 8
GC, CO	0	—	—	81 ± 2	10.3 ± 0.6	—	7.9 ± 0.5

Note. Primed parameters refer to the fast phase, unprimed to the slow. Note the different units for k'_a and k_a.

accounts for more than half of the association. Since the two phases are so distinct, we analyzed them separately to produce phenomenological parameters summarized in Table 1.

Other pertinent observations for our solution conditions include the following: Although there was no very dramatic change in the absorption spectrum, there was a reproducible small shift in the Soret absorption band from 424 nm down to 421 nm. The time required for completion of this shift after YC-1 was added to a solution of pre-mixed GC-CO was 5 to 10 seconds, depending on the YC-1 concentration. We could not measure how long it takes YC-1 to bind in the absence of CO, since we did not see a change in the spectrum; it could be even longer. The lack of a spectral shift in the absence of CO suggests either that the affinity for YC-1 is lower, or that binding in the absence of CO is at a non-heme site. Finally, preparation of GC-CO-(YC-1) mixtures at several [CO] showed that affinity of GC for CO increased modestly in the presence of YC-1, about 20-fold, as predicted (16).

What could account for the differences between the present observations and those reported previously (17), which were obtained using stopped-flow methods? The stopped-flow kinetic measurements had excellent time resolution, displaying data points at 1 ms intervals; still that was insufficient to detect the fast process directly, at least for the fairly high CO concentrations they investigated. Nevertheless, any fast process accounting for 60 to 75% of the total absorbance change could scarcely have gone unrecognized. There would have been substantial "missing amplitude" due to the combination that occurred during the instrumental dead time. Kineticists using stopped-flow methods are alert to dead-time problems. Consequently, it is incumbent on us to explain why the fast process must have been smaller or absent altogether. We can suggest one difference in solution composition that could well have resulted in a smaller fraction for the fast process. According to the protocol described, the earlier study did not use conditions actually present during catalysis; in particular the solutions did not include substrate (GTP) and product (cGMP). We previously showed that GTP/cGMP have profound effects on ligand binding kinetics in the GC system (18). When we made a measurement by flash photolysis in the absence of GTP/cGMP, we found the fast process was still present, but reduced in amplitude to about 20–25% of the total. We should not speculate exactly what the limit of detection may have been in the earlier measurements. For purposes of this discussion, let us assume that measurement precision was very good in the mixing experiment, and the experiments did conclusively show, as implied, that no fast process at all was missed. If this assumption is valid, then there are very interesting consequences to explore.

A genuine difference between mixing and flash perturbation experiments in the presence of YC-1 would suggest that the protein exists in two forms, with the fraction of each present depending upon CO and YC-1. The forms must interconvert slowly compared to the time required for ligand association and dissociation. The situation is reminiscent of the two forms of hemoglobin: A fast laser photolysis measurement characterizes ligand bimolecular recombination with the fast reacting "R" or "liganded" state (as well as geminate recombination, not considered here). However, mixing deliganded protein with CO detects and characterizes only the slow reacting "T" or "unliganded" state. Neither experiment is incorrect; together they help us appreciate the richness of protein behavior. If YC-1 reacts significantly only with carboxyGC, as spectroscopic data indicate, then stopped-flow experiments certainly could not measure the effect of YC-1 on CO combination. Note that whatever their origin, the phases we observe both show CO dissociation rate constants that are extraordinarily high for heme species with 6-coordinate imidazole-iron-CO (20). They are in a range that suggests that dissociation proceeds by way of a 5-coordinate intermediate (22), as we suggested previously (23).

A key question is, exactly where is YC-1 bound in the protein and what sort of spectral change should one expect? It is clear that YC-1 is quite hydrophobic; it has to be dissolved in DMSO, then added to the aqueous phase. It should have considerable propensity to be incorporated into the protein at one or more sites, perhaps not very specific. Such generalized hydrophobic interaction should not affect the spectrum of the heme center. This might be what is happening in the absence of CO. Mild, heme-ligation-independent activation of GC by hydrophobic molecules has been reported

(8). We note, however, that YC-1 has two nitrogens, each capable of donating a nonbonding pair of electrons to coordinate with the heme iron. This apparently does not happen to provide a sixth ligand (a distal ligand) in the absence of CO, as it should be accompanied by a large spectral shift, which is not observed. For the case of GC-CO, however, replacement of the proximal base β_1H105 by YC-1 with retention of CO as the distal ligand should cause only a small spectral shift in the Soret region, as we observed, since there will be no change in the coordination number of the heme iron, and one nitrogenous base is simply replaced by another. Most interestingly, this hypothesis provides a free proximal base. Consequently, this model explains both the six-coordinate spectrum and the high enzyme activity, without abandoning the notion that freeing the proximal base may be essential to enzyme activity (24), an hypothesis that survived very well one specific test (25). It seems that the effect of YC-1 may be to replace the proximal base or to stretch the proximal bond considerably so as to render it highly labile. In either case, only a small shift in the Soret band is expected (26).

The proposal that YC-1 in GC-CO may replace the normal proximal base explains: enzyme activation that matches that of NO, prior direct evidence for the need to break the proximal bond to achieve maximal activation (25), the small shift we observed in the position of the Soret absorption peak, and a dramatic effect on ligation kinetics. The idea is largely independent of mechanistic details of CO ligation or the differences in the observations of the stopped-flow and flash photolysis experiments. Unraveling these will provide insight into the temporal sequence by which the reactions occur. These issues will be addressed in a future report that will elaborate on the kinetics observed under a variety of conditions.

In summary, kinetic, spectroscopic, and enzymatic data for GC in the presence and absence of YC-1 are explained satisfactorily by a model that requires free or highly labile proximal base. Although we don't discuss GC-NO here, the model applies well to both nitrosyl and carboxy derivatives of soluble guanylate cyclase.

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REFERENCES

1. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chandhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9265–9269.
2. Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988) *Nature* **336,** 385–388.
3. Moncada, S., and Higgs, E. A. (1995) *FASEB* **9,** 1319–1330.
4. Waldman, S. A., and Murad, F. (1987) *Pharmacol. Rev.* **39,** 163–196.
5. Garbers, D. L., and Lowe, D. G. (1994) *J. Biol. Chem.* **269,** 30741–30744.
6. Humbert, P., Niroomand, F., Fischer, G., Mayer, B., Koesling, D., Hinsch, K.-D., Gausepohl, H., Frank, R., Schultz, G., and Böhme, E. (1990) *Eur. J. Biochem.* **190,** 273–278.
7. Stone, J. R., and Marletta, M. A. (1996) *Biochemistry* **35,** 1094–1099.
8. Ignarro, L. J. (1989) *Semin. Hematol.* **26,** 63–76.
9. Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Böhme, E., Schultz, G., and Koesling, D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2592–2596.
10. Zhao, Y., Schelvis, J. P. M., Babcock, G. T., and Marletta, M. A. (1998) *Biochemistry* **37,** 4502–4509.
11. Senter, P. D., Eckstein, F., Mülsch, A., and Böhme, E. (1983) *J. Biol. Chem.* **258,** 6741–6745.
12. Traylor, T. G., Duprat, A. F., and Sharma, V. S. (1993) *J. Am. Chem. Soc.* **115,** 810–811.
13. Brüne, B., and Ullrich, V. (1987) *Mol. Pharmacol.* **32,** 497–504.
14. Stone, J. R., and Marletta, M. A. (1994) *Biochemistry* **33,** 5636–5640.
15. Ko, F.-N., Wu, C.-C., Kuo, S.-C., Lee, F.-Y., and Teng, C.-M. (1994) *Blood* **84,** 4226–4233.
16. Friebe, A., and Koesling, D. (1998) *Mol. Pharmacol.* **53,** 123–127.
17. Stone, J. R., and Marletta, M. A. (1998) *Chem. Biol.* **5,** 255–261.
18. Kharitonov, V. G., Russwarm, M., Magde, D., Sharma, V. S., and Koesling, D. (1997) *Biochem. Biophys. Res. Commun.* **239,** 284–286.
19. Walda, K. N., Liu, X. Y., Sharma, V. S., and Magde, D. (1994) *Biochemistry* **33,** 2198–2209.
20. Li, T., Quillin, M. L., Phillips, J. S., and Olson, J. S. (1994) *Biochemistry* **33,** 1443–1446.
21. Stone, J. R., and Marletta, M. A. (1995) *Biochemistry* **34,** 16397–16403.
22. Traylor, T. G., Campbell, D., Sharma, V., and Geibel, J. (1979) *J. Am. Chem. Soc.* **101,** 5376–5383.
23. Kharitonov, V. G., Sharma, V. S., Pilz, R. B., Magde, D., and Koesling, D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2568–2571.
24. Traylor, T. G., Duprat, A. F., and Sharma, V. S. (1993) *J. Am. Chem. Soc.* **115,** 810–811.
25. Dierks, E. A., Hu, S., Vogel, K. M., Yu, A. E., Spiro, T. G., and Burstyn, J. N. (1997) *J. Am. Chem. Soc.* **119,** 7316–7323.
26. Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., and Simon, S. R. (1976) *Biochemistry* **15,** 378–387.