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Dissociation of Nitric Oxide from Soluble Guanylate Cyclase

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Kinetic studies of soluble guanylate cyclase complexed with nitric oxide prove that NO dissociation in the presence of the substrate GTP and Mg^{2+} is as much as 50 times faster than in their absence. In the presence of those two reagents the dissociation rate constant is $k_{obs} = 0.04 \pm 0.01\ s^{-1}$ at 20 °C, which is by far the fastest NO dissociation rate constant ever reported for a ferrous heme protein. Extrapolated to 37 °C, this corresponds to a half life of about 5 s for NO dissociation from soluble guanylate cyclase at physiological conditions, which is presumably fast enough to account for deactivation of the enzyme in biological systems. Dissociation rate constants are also reported for a variety of other reagent conditions.

Soluble guanylate cyclase (GC) is a heme protein that catalyses conversion of guanosine 5′-triphosphate (GTP) to cyclic guanosine 3′,5′-monophosphate (cGMP) (1-3). In the resting, or basal, state, the heme iron is coordinated to a (proximal) histidine, producing a five-coordinate iron. Activity is markedly increased when nitric oxide is coordinated to the distal side of the heme iron. Binding NO causes rupture of the proximal bond, so that the heme iron is again five-coordinate, but with distinctly different spectra and properties. This rupture of the proximal bond presumably results in a significant conformational change that enhances reactivity. It is possible the proximal histidine itself plays some role in catalysis; but almost nothing is known about details of the behavior of GC. This letter addresses one critical detail, the rate at which NO is released from the enzyme, which is expected to be an important factor in "down regulating" the enzyme, that is, turning off its activity.

Considerable experience with ligation reactions in other heme proteins leads to two confident predictions for the NO reactivity: 1) Association of NO with a five-coordinate iron should be close to diffusion controlled. 2) Dissociation of NO from a five-coordinate iron is generally quite slow. The first prediction appears to hold for GC; association kinetics were reported to be very complex but quite fast (4). As for the second prediction, it is already clear that GC behavior contradicts that prediction. We recently reported NO dissociation rate constants for several nitrosyl heme proteins and model hemes, including GC (5). We inferred that at 37 °C the half-life for NO dissociation from GC-NO could be as short as 2 min, allowing for a generous margin of error in a value that more directly suggests about 4 min. Any such value is surprisingly fast (by orders of magnitude) for a five-coordinate NO heme. However, even two minutes still seemed slower than would be necessary to account for deactivation of the enzyme in biological systems. Even though there is no definitive data for the speed of response *in vivo,* deactivation is generally thought to be in the range of a few seconds, based on inferences from whole-organ physiology (6). It may be that additional mechanisms exist for down regulating GC activity without dissociating NO; but before jumping to that conclusion, we decided to look for special conditions that might enhance the NO dissociation rate even further. We had noticed over several months that enzyme activity diminished quickly under certain experimental conditions, and this prompted a systematic study and suggested what variables should be explored. We present here the result of studies that indicate that in the presence of the substrate GTP and Mg^{2+}, dissociation of NO from GC-NO is much faster even than the fast dissociation we had obtained without those reagents. Kinetic data point to a half-life of about 5 s at 37 °C. Such a high rate of NO dissociation is unparalleled in any previously studied hemeprotein, and has significant implications for the mechanism of deactivation of guanylate cyclase, an important and ubiquitous enzyme.

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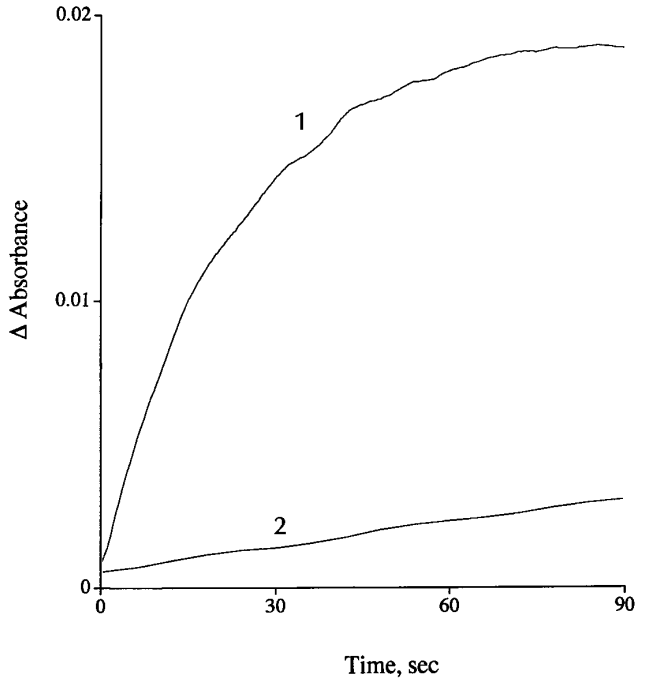

FIG. 1. Reaction time courses for NO dissociation from GC-NO in the presence (1) and in the absence (2) of GTP and Mg^{2+}. For experimental conditions, see Table 1.

MATERIALS AND METHODS

Soluble guanylate cyclase was purified from bovine lung and characterized spectroscopically as described previously (1). Enzyme activity was increased ~200-fold by reaction with NO. Kinetic experiments were made using a double-beam spectrophotometer at 20 °C. Solutions containing the enzyme, dithionite, NO, and/or other reagents were prepared in the usual manner (7). Dissociation was monitored by mixing the enzyme solution with a solution of scavenger, either HbO_2 or MbO_2, which at high enough concentration will react immediately and irreversibly with any free NO (5). In a typical experiment, to deoxygenated buffer in a spectrophotometer cell a stock solution of GC was added that would produce a final protein concentration of 1 μM. To this was added a volume of 200 μM NO solution calculated to yield a final NO concentration of 5 μM. Excess NO was removed by degassing with argon for five minutes. The cell was then opened to air and the scavenger was added to a final concentration of 5 μM. Subsequent absorption changes as NO dissociated and combined with scavenger were monitored at 435 nm. The concentration of scavenger was varied over the range 2-5 μM in order to prove that dissociation was the rate-limiting step being measured.

RESULTS AND DISCUSSION

Reaction time courses for NO dissociation from GC-NO appeared as good single exponential traces. Two examples that illustrate strikingly different behavior are shown in Figure 1. Dissociation rate constants observed for a variety of conditions are collected in Table 1. The first entry listed is the same as the result obtained previously (5) for GC-NO in the absence of substrate, namely 0.0007 s^{-1}. Certain reaction conditions produce much faster dissociation rate constants; and others lead to an intermediate result. Most of the table entries simply list the dissociation rate as "slow." This means that the half-life is more than 10 min. It is probably very similar to that of the first entry; but it is listed simply as "slow" because there are severe experimental difficulties in studying those very slow reactions in detail with high precision. The presence of Mg^{2+}, required for enzyme activity, precludes the addition of EDTA, and it is very difficult or impossible to maintain a stable baseline over long times without EDTA for solutions of either MbO_2 or HbO_2, which are needed as the NO scavenger. Fortunately, it is the fast dissociations that are of interest in this study.

The rate constant for NO dissociation from GC-NO in the presence of Mg^{2+} and GTP is the fastest in Table I and the fastest dissociation yet measured for any nitrosyl heme protein. Adding cGMP gives a slightly lower dissociation rate, but the difference is not statistically significant. We averaged the two numbers to obtain the result quoted in the abstract, 0.04 s^{-1}. Extrapolated to 37 °C with approximately a doubling every 10 °C, this corresponds to a half-life for dissociation at physiological conditions of about five seconds, which is short enough to make down regulation of GC by NO release much more plausible than it seemed previously.

The question remains exactly which species is most responsible for the rate enhancement. In the presence of substrate and the active enzyme, product species will gradually accumulate. Can any of these affect NO dissociation and provide feedback, reducing enzyme activity as products accumulate? The data show that cGMP or pyrophosphate are not effective, either by themselves or in the presence of Mg^{2+}. The ion Mg^{2+} is essential, according to Table I; yet Mg^{2+} by itself is not

TABLE 1

Rate Constants Observed for NO Dissociation from GCNO at 20 °C and pH 7.4 in 50 mM Triethanolamine, 3mM DTT and 0.5 mg/mL BSA, with $[MbO_2]$ = 2-5 μM

Conditions	k_{obs}/s^{-1}
GCNO	6-8 × 10^{-4}
GCNO + Mg^{2+} + GTP + cGMP	0.03 ± 0.01
GCNO + Mg^{2+} + GTP	0.05 ± 0.01
GCNO + Mg^{2+} + γ-sGTP + cGMP	slow
GCNO + Mg^{2+} + cGMP	slow
GCNO + Mg^{2+} + pyrophosphate	slow
GCNO + cGMP	slow
GCNO + cGMP + EDTA	slow
GCNO + pyrophosphate	slow
GCNO + cGMP + pyrophosphate	slow
GCNO + GTP	0.005 ± 0.002
GCNO + GTP + cGMP	0.005 ± 0.002
GCNO + GTP + cGMP + EDTA	slow

Note. When indicated, $[Mg^{2+}]$ = 3 mM; [GTP or γ-sGTP] = 0.5 mM; [cGMP] = 1 mM; [EDTA] = 0.5 M; [pyrophosphate] = 0.5 mM.

sufficient. Even given Mg^{2+}, the GTP analog guanosine 5′-O-(3-thiotriphosphate), γ-sGTP, is not effective. It is clear that GTP and Mg^{2+} are key; anything else has at most a minor effect. The one intermediate case, for which the dissociation constant is 0.005 s^{-1}, is that of GTP by itself or with added cGMP, but without Mg^{2+}. Even in this case, adding EDTA causes the reaction to become "slow." This could be interpreted as EDTA scavenging trace amounts of Mg^{2+} impurity or some other metal ion that could substitute, perhaps not as effectively, for Mg^{2+}.

Two mechanisms may be proposed to account for enhancement of NO dissociation in GC. There could be distal-side strain at the NO itself. Such strain has only a very modest effect in myoglobins with different residues in the distal pocket; but the situation might be different in GC, if the lack of a proximal bond allows greater interaction with the distal environment. The fact that a recent study of Raman frequency shifts demonstrated that binding GTP has a direct influence on NO vibrational frequencies (8) favors this hypothesis. The same study, however, showed virtually similar effects on the Raman spectrum for GTP and cGMP, and yet the dissociation rate constant with cGMP alone is different from that with GTP alone. Another hypothesis would consider a possible role for the proximal histidine. Changes there often have profound effects. In particular, NO dissociation is much faster when histidine is present in the proximal position, so that even a small, steady state concentration of six-coordinate iron could have a significant effect. However, GC is usually considered to have no bond to the proximal histidine when activated by NO, as judged both from the visible spectrum and from two Raman studies that focused expressly on this point (9,10). On the other hand, one EPR study (11) suggested a small admixture of six-coordinated iron and a study of association kinetics (4) invoked a complicated reaction scheme that implied a small fraction of six-coordinate iron at equilibrium. At present, it is best to regard the mechanism of enhanced dissociation as an open question, but it is noteworthy that GC shows again how versatile heme proteins really are: when the GC enzyme required an exceptional dissociation for NO, evolution was able to select for that property.

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REFERENCES

1. Humbert, P., Niroomand, F., Fischer, G., Mayer, B., Koesling, D., Hinsch, K.-D., Gausepohl, H., Frank, R., Schultz, G., and Bohme, E. (1990) *Eur. J. Biochem.* **190,** 273–278.
2. Schmidt, H. H. H. W. (1992) *FEBS Lett.* **307,** 102–107.
3. Stone, J. R., and Marletta, M. A. (1994) *Biochemistry* **33,** 5636–5640.
4. Stone, J. R., and Marletta, M. A. (1996) *Biochemistry* **35,** 1094–1099.
5. Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997) *Biochemistry* **36,** 6814–6818.
6. Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987) *Nature* **327,** 524–526.
7. Kharitonov, V. G., Sundquist, A. R., and Sharma, V. S. (1995) *J. Biol. Chem.* **270,** 28158–28164.
8. Tomita, T., Ogura, T., Tsuyama, S., Imai, Y., and Kitagawa, T. *Biochemistry*, in press.
9. Deinum, G., Stone, J. R., Babcock, G. T., and Marletta, M. A. (1996) *Biochemistry* **35,** 1540–1547.
10. Yu, A. E., Hu, S., Spiro, T. G., and Burstyn, J. N. (1994) *J. Am. Chem. Soc.* **116,** 4117–4118.
11. Stone, J. R., Sands, R. H., Dunham, W. R., and Marletta, M. A. (1995) *Biochem. Biophys. Res. Commun.* **207,** 572–577.