HEME PROTEIN-DIIMIDE COMPLEXES: POSSIBLE INTERMEDIATES IN BIOLOGICAL NITROGEN FIXATION

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A suggested pathway for nitrogen fixation is the stepwise reduction of nitrogen through diimide and hydrazine to produce ammonia.[1, 2] Hydrazine is toxic to some nitrogen-fixing systems[3] and neither free hydrazine nor diimide has been demonstrated to be present or involved in nitrogen-fixing systems.[3, 4] It therefore seems likely that if this diimide-hydrazine pathway is used by nitrogen-fixing organisms, the reduction intermediates remain bound to an enzyme, probably at a metal site.[2] Because legoglobin, the heme pigment in the nitrogen-fixing soybean root nodules, is reported to bind nitrogen,[5] we have sought diimide complexes of legoglobin and other heme compounds.

We have found that such complexes form readily and are stable for periods ranging from minutes to hours depending upon the structure of the heme protein. Typically, solutions of hemoglobin, legoglobin, whale myoglobin, or chlorohemin in 1 : 1 tris-hydrochloric acid buffers (pH = 8.1) were made 10^{-4} M in the heme, reduced with sodium dithionite,[6] and treated with potassium azodiformate in 40–700 per cent molar excess.[7] The spectrum of the reduced heme was immediately replaced by a typical hemochrome spectrum similar to that observed when pyridine is added in large amounts.[10] Visible spectra of myoglobin before and after reduction with dithionite and after addition of potassium azodiformate are shown in Figure 1. Spectra of the other complexes are tabulated in Table 1.
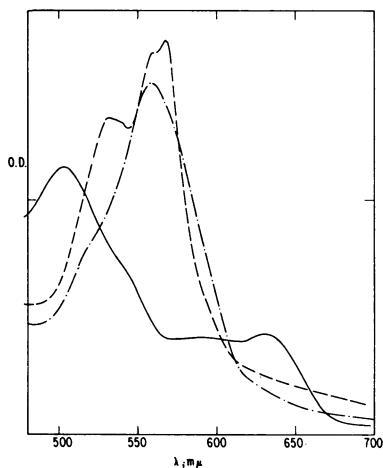

FIG. 1.—Spectra of myoglobin in pH = 8 tris buffer. ————, Metmyoglobin; — · —, reduced myoglobin; – – –, myoglobin–diimide complex.

These spectra revert to those of the reduced hemes in 10–60 minutes with the three heme proteins tested. However, the hemochrome spectrum of the complex derived from ferroprotoporphyrin decreases in intensity less than one per cent in

one hour in a nitrogen atmosphere and somewhat faster in the presence of oxygen. When a large amount of pyridine is added to the latter complex, the pyridine hemochrome[10] spectrum appears immediately. This conversion is accompanied by nitrogen evolution, as demonstrated by mass spectral analysis. Methylene blue is reduced by this complex and causes the hemochrome spectrum to revert to that of the ferroprotoporphyrin combined with the spectrum of the excess methylene blue.

We have demonstrated that neither hydrazine nor ammonia form complexes with protein-bound ferroprotoporphyrin at concentrations which might arise from diimide decomposition.[9] Furthermore, the hydrazine hemochrome differs from diimide complex (Table 1). No spectral change occurred when ferroprotoporphyrin

TABLE 1

SPECTRA OF DIIMIDE AND OTHER HEMOCHROMES IN pH = 8 TRIS BUFFER

Heme	Added ligand[a]	$\lambda_{max}(\beta)$[b]	λ_{min}[b]	$\lambda_{max}(\alpha)$[b]	ϵ_{ratio}[c]
Myoglobin	N_2H_2	530	543	567, 559[d]	1.32
Hemoglobin	N_2H_2	528	540	557	1.33
Legoglobin	N_2H_2	522	535	552	1.3
Ferroprotoporphyrin IX	N_2H_2[e]	530	543	561	1.23
"	N_2H_4[f]	526	537	555	1.73
"	Pyridine[g]	523	539	554	1.54

[a] The reduced forms of all the heme compounds listed have broad maxima at about 555.
[b] Mμ.
[c] The ratio of the OD for the α band to that of the β band.
[d] E. Bechtold (*Biochem. Z.*, 331, 427 (1942)) observed a transitory shoulder at 566 when metmyoglobin was treated with hydrazine.
[e] Formed by adding potassium azodiformate to solutions of ferroprotoporphyrin IX.
[f] Formed by adding excess hydrazine to solutions of either chlorohemin or ferroprotoporphyrin IX.
[g] Eighty per cent in pyridine, 20 per cent in tris buffer pH 8.0.

was treated with potassium azodiformate at pH > 13 where diimide formation is negligibly slow.[8] When this solution was acidified, the hemochrome spectrum appeared immediately. Conversely, treatment of ferroprotoporphyrin with potassium azodiformate at pH = 8.1 produced the hemochrome spectrum which was not discharged when the solution was brought to pH = 13. Therefore the complex does not involve azodiformate as acid, monoanion, or dianion.

The reducing power of the myoglobin-diimide complex was demonstrated as follows: myoglobin (0.26 μmole in 3.0 ml of pH = 8 tris buffer) was titrated with sodium dithionite and the slight excess dithionite discharged with methylene blue. Potassium azodiformate (9 μmoles) was added to form the complex. After three minutes, buffered methylene blue (0.072 μmole) solution was added. The hemochrome spectrum reverted to that of reduced myoglobin and 4.4×10^{-2} μmoles of methylene blue was consumed. When the experiment was repeated, omitting either potassium azodiformate or myoglobin, no or negligible ($<10^{-3}$ μmoles) methylene blue was consumed. Similarly, when potassium azodiformate was added to chlorohemin at pH = 8, the hemochrome spectrum appeared immediately and, after standing ten minutes, the solution still reduced methylene blue. Methylene blue was not reduced when chlorohemin was omitted.

These results indicate that diimide is stabilized by complexing with ferropor-phyrins.[11] Possible structures (I and II) of the complexes are analogous to those proposed for bound oxygen.[12, 13]

Although we prefer structure (I), a decision between these structures must await X-ray crystallographic studies.

These results suggest that such diimide complexes as that with legoglobin might be the first intermediate in nitrogen fixation. Experiments to test the possible

(I)   (II)
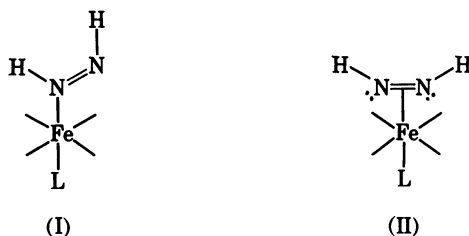
involvement of the legoglobin-diimide complex in nitrogen fixation in soybean root nodules and studies of other diimide-metal complexes are in progress.

[1] Bauer, N. A., *Nature,* **188,** 471 (1960).

[2] Abel, K., *Phytochemistry,* **2,** 429 (1963).

[3] Garcia-Rivera, J., and R. H. Burris, *Arch. Biochem. Biophys.,* **119,** 167 (1967).

[4] Stewart, W. D. P., *Nitrogen Fixation in Plants* (London: Athlone Press, 1966), p. 102.

[5] Abel, K., Bauer, N., and J. T. Spence, *Arch. Biochem. Biophys.,* **100,** 339 (1963).

[6] The sodium dithionite reduction may be omitted because the diimide which forms will reduce the heme proteins and subsequently form the complex.

[7] At a pH = 8, potassium azodiformate decomposes in a few milliseconds to produce diimide,[8] which in turn disappears in about 1 min.[9]

[8] King, C. V., *J. Am. Chem. Soc.,* **62,** 379 (1940).

[9] Hünig, S., H. R. Müller, and W. Thier, *Angew Chem.,* (Int. ed)., **4,** 271 (1965). Whereas 10^{-3} M azodiformate gives the diimide myoglobin complex, at least 10^{-1} M hydrazine is required to produce any spectral change in ferromyoglobin.

[10] Falk, J. E., *Porphyrins and Metalloporphyrins* (New York: Elsevier Publishing Company, 1964), p. 240.

[11] Diels, O., and W. Koll, *Ann. Chem.,* **443,** 262 (1925) suggested the possibility of stabilizing diimide by metal complexation but did not pursue this idea.

[12] Pauling, L., and C. D. Coryell, these PROCEEDINGS, **22,** 159 (1936).

[13] Griffith, J. S., *Proc. Roy. Soc. (London), Ser. A.,* **235,** 23 (1956).