Phytochemistry, 1963, Vol. 2, pp. 429 to 435. Pergamon Press Ltd. Printed in England

THE PHYSICAL AND CHEMICAL BASIS OF SYMBIOTIC NITROGEN FIXATION: RECENT ADVANCES

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(*Received* 5 *March* 1963)

INTRODUCTION

RECENTLY we reported the first direct *in vitro* evidence that legoglobin (also referred to as "leghemoglobin") is the site at which nitrogen fixation occurs in the symbiotic system.[1] In reporting this evidence, no correlation of these findings was made with data reported from other laboratories. It is the purpose of this discussion to review the work of other people in this field, thereby demonstrating a direct correlation of our findings with previously reported data and suggest a probable mechanism for the first steps in nitrogen fixation.

REVIEW

Properties of Legoglobin

An excellent review of biological nitrogen fixation studies covering the period to 1947 was made by Wilson and Burris.[2] At that time they pointed out that the nitrogen fixation process appeared to be significantly different in the symbiotic system from that in the free-living nitrogen-fixing micro-organisms. As an example, they mentioned the then recent findings of Virtanen *et al.*[3, 4a] that two heme proteins are invariably present in root nodules which are actively fixing nitrogen, but are not found in the free-living nitrogen-fixing micro-organisms. These proteins were called "leghemoglobins" by Virtanen and his associates. Ellfolk and Virtanen[4b] studied the two components of ferrileghemoglobin more closely and found them to have unusual acidic isoelectric points of 4·4 and 4·7 . A molecular weight of ~ 17,000 was found for the component with I.P. 4·4.[4c] Subsequently, Ellfolk[5, 6] crystallized the two ferrileghemoglobins and determined that the fast electrophoretic component has a molecular weight between 16,695 (determined from amino acid composition) and 17,500 (determined from the iron content), is spherical, and has an isionic point at pH 4·5 for the apoprotein, while the slow electrophoretic component has a molecular weight between 15,429 (amino acid composition) and 19,500 (iron content), is assymmetric, and has an isoionic point at pH 5·6 for the apoprotein. When the two ferriporphyrin carboxyl groups present in the whole leghemoglobin components are included, the measured values for the

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[1] K. ABEL, N. BAUER and J. T. SPENCE, *Arch. Biochem. Biophys.* **100**, 338 (1963).
[2] P. W. WILSON and R. H. BURRIS, *Bacteriol. Rev.* **11**, 41 (1947).
[3] A. I. VIRTANEN, J. JORMA, H. LINKOLA and A. LINNASALMI, *Acta Chem. Scand.* **1**, 90 (1947).
[4a] A. I. VIRTANEN, J. ERKAMA and H. LINKOLA, *Acta Chem. Scand.* **1**, 861 (1947).
[b] N. ELLFOLK and A. I. VIRTANEN, *Acta Chem. Scand.* **4**, 1014 (1950).
[c] N. ELLFOLK and A. I. VIRTANEN, *Acta Chem. Scand.* **6**, 411 (1952).
[5] N. ELLFOLK, *Acta Chem. Scand.* **15**, 545 (1961).
[6] N. ELLFOLK, *Acta Chem. Scand.* **13**, 596 (1959).

isoelectric points 4·4 and 4·7 are in good agreement with the isoionic points calculated for the apoproteins. He also determined that there are no sulfur-containing amino acids in either component and suggested from fatty acid complex studies and the rapid autoxidation of his preparations that the heme group is exposed on the surface of the molecule[7,8] as opposed to myoglobin and hemoglobin.[9] It was subsequently shown by Abel and Bauer[10] that the heme group in ferrolegoglobin is not exposed; but that autoxidation in air results in molecular rearrangement with subsequent, irreversible exposure of the heme. (In the absence of sulfur-containing amino acids, histidine has been implicated as being primarily responsible for maintaining the globular structure of proteins.[11] The legoglobins are extremely low in histidine[4c,6] so that molecular rearrangement would be expected to occur even under mild conditions.) The legoglobin has been found to be diffused through the cytoplasm of the nodule cells and is not located within the infecting bacteria.[12]

Correlations of Legoglobin and Nitrogen Fixation

Following Virtanen's[3,5] observations that symbiotic nitrogen fixation does not occur in the absence of legoglobin, several investigators confirmed his results.[13,14] Almost 10 yr after Virtanen's work, Hamilton *et al.*[15] demonstrated that the legoglobin absorption spectrum of cell-free nodule extracts prepared under hydrogen changed from a typical oxygenated (ferrolegoglobin) spectrum to the oxidized (ferrilegoglobin) spectrum upon addition of nitrogen and this was reversed when hydrogen was re-admitted. Although this observation has not yet been confirmed, it is of interest to note that the properties of legoglobin extracted under hydrogen are considerably different from the properties of legoglobin extracted under inert gases. For example, the autoxidation rates and electrophoretic mobilities[10] are very different, and of equal (or greater) importance, the gas complexing characteristics change. Early work with legoglobin extracted by conventional techniques indicated that legoglobin had only a slight affinity for nitrogen.[16] Although it was shown that this affinity was greater than anticipated from simple solubility phenomena, it was only slightly greater than for hemoglobin and myoglobin, both of which also show a greater affinity for nitrogen than can be explained on the basis of simple solubility.[17] As a result, this affinity for nitrogen was described as a "secondary" attachment as opposed to direct attachment at the heme group. We have subsequently demonstrated that, when exposed to air, the heme group of hydrogen-extracted legoglobin preferentially complexes with nitrogen rather than oxygen,[1] that attachment occurs directly at the heme group, and that this legoglobin–nitrogen complex cannot be broken by simply reducing the partial pressure of nitrogen. This is the first *in vitro* evidence that legoglobin has a nitrogen attachment site of sufficient strength to be indicative of chemical bonding rather than a simple physical "fit" or solution effect.

The unusual effect of a specific hydrogen treatment on the properties of legoglobin is less

[7] N. ELLFOLK, *Acta Chem. Scand.* **15**, 975 (1961).
[8] N. ELLFOLK and K. LEVIN, *Acta Chem. Scand.* **15**, 444 (1961).
[9] J. C. KENDREW, *Sci. American* December, p. 96 (1961).
[10] K. ABEL and N. BAUER, *Arch. Biochem. Biophys.* **99**, 8 (1962).
[11] E. L. SMITH, *J. Biol. Chem.* **233**, 1392 (1958).
[12] J. D. SMITH, *Biochem. J.* **44**, 585 (1949).
[13] F. J. BERGERSEN, *Biochim. Biophys. Acta* **50**, 576 (1961).
[14] J. E. FALK, C. A. APPLEBY and R. J. PORRA, in *Utilization of Nitrogen and Its Compounds by Plants*, Symposia of the Society for Experimental Biology, Cambridge University Press, Vol. XIII, 73 (1959).
[15] P. B. HAMILTON, A. L. SHUG and P. W. WILSON, *Proc. Nat. Acad. Sci., U.S.* **43**, 297 (1957).
[16] R. G. MORTIMER and N. BAUER, *J. Phys. Chem.* **64**, 387 (1960).
[17] D. D. VAN SLYKE, R. T. DILLON and R. MARGARIA, *J. Biol. Chem.* **105**, 571 (1934).

surprising when it is realized that Wilson *et al.*[18, 19] several years previous to the discovering of legoglobin found that: (a) hydrogen is evolved from soybean root nodules in the absence of a readily available source of nitrogen, (b) hydrogen inhibits nitrogen fixation, (c) nitrogen inhibits hydrogen evolution, and (d) carbon monoxide and nitrous oxide inhibit both hydrogen evolution and nitrogen fixation. (Interestingly, Wilson[20] has just recently reported that hydrogen is evolved by nitrogen-fixing bacteria and that hydrogen inhibits nitrogen fixation in this system as well, indicating that the two systems may be more closely related than was previously thought). He also found that whereas nitrogen enhances the exchange reaction $H_2 + D_2 \rightarrow 2HD$, nitrous oxide and carbon monoxide inhibit the exchange reaction. The only significant difference found between nitrous oxide and carbon monoxide was that nitrous oxide also inhibited hydrogen evolution whereas carbon monoxide did not. The fact that both carbon monoxide and nitrous oxide form complexes with heme proteins leads to the obvious conclusion that legoglobin is directly concerned with the first steps in nitrogen fixation.

Several hypotheses have been suggested to account for the presence of legoglobin and these are listed below.

One of the earliest hypotheses regarding the function of legoglobin was made by Kasugai *et al.*[21], and independently by Little and Burris.[22] This hypothesis ascribed a respiratory role to legoglobin and was probably made because of the apparent similarities between legoglobin and the vertebrate heme proteins. It was implied that an increased respiratory function would indirectly affect the rate of nitrogen fixation. No evidence has been found to substantiate this view.[23] Furthermore, heme proteins that are similar in many respects to legoglobin have since been isolated from other organisms and these heme proteins have been shown to have no respiratory role.[24, 25]

Virtanen[26, 27] was the first to suggest that fixation was accompanied by a valence change of the iron in legoglobin. Later Virtanen *et al.*[28] and Pethica *et al.*[29] suggested that hydroxylamine formation would possibly be the key step in nitrogen fixation. Pethica postulated that hydrogenase sites exist on one portion of the legoglobin molecule and nitrogenase sites on another portion with the first step being an oxidative formation of hydroxyl radicals by legoglobin. Bauer[30] tended to concur with part of this view but indicated from both an experimental and theoretical viewpoint that both the hydrogenase and nitrogenase sites would be located on or near the same position in legoglobin. Virtanen demonstrated that legoglobin will catalyze the decomposition of hydroxylamine to ammonia, and ammonia has been established as the intermediate in nitrogen fixation.[31] In our own laboratories[32] Virtanen's

[18] P. W. WILSON, *The Biochemistry of Symbiotic Nitrogen Fixation.* University of Wisconsin Press, Madison (1930).
[19] E. R. EBERSIDE, C. GUTLENTAG and P. W. WILSON, *Arch. Biochem.* **3**, 1399 (1944).
[20] F. H. GRAU and P. W. WILSON, *J. Bacteriol.* **83**, 490 (1962).
[21] S. KASUGAI, H. KUBO and K. TSUMIMIRA, *J. Agr. Chem. Soc., Japan* **19**, 765 (1948).
[22] H. N. LITTLE and R. H. BURRIS, *J. Am. Chem. Soc.* **69**, 838 (1947).
[23] J. D. SMITH, *Biochem. J.* **44**, 591 (1949).
[24] H. E. DAVENPORT, *Proc. Roy. Soc.*, B. **136**, 255 (1949).
[25] H. E. DAVENPORT, *Proc. Roy. Soc.*, B. **136**, 271 (1949).
[26] A. I. VIRTANEN and T. LAINE, *Suomen Kemistilehti B* **18**, 39 (1945).
[27] A. I. VIRTANEN, *Nature* **155**, 747 (1945).
[28] A. I. VIRTANEN, A. KEMPI and E. SALMANOJA, *Acta Chem. Scand.* **8**, 1729 (1954).
[29] B. A. PETHICA, E. R. ROBERTS and E. R. S. WINTER, *Biochim. Biophys. Acta* **14**, 85 (1954).
[30] N. BAUER, *Nature* **188**, 471 (1960).
[31] R. H. BURRIS in *Symposium on Inorganic Nitrogen Metabolism*, p. 317. John Hopkins University Press, Baltimore (1956).
[32] D. O. EDLUND, *Hemoglobin–hydroxylamine Interaction in Aqueous Solution*, Thesis, Utah State University, Logan, Utah (1960).

observations have been confirmed. However, the reaction is much more complicated than he indicated with nitrous oxide being one of several products and very different mechanisms being in evidence depending upon whether the system is aerobic or anaerobic.

A reductive first step has been suggested by Bach[33] and by Hoch *et al.*[34], but in neither case was reduction suggested to occur on a legoglobin site. Instead, they suggested that nitrogen was attached on the surface of an unspecified enzyme at a site having two metal groups in close proximity capable of simultaneously losing one electron each to nitrogen, eventually leading to the formation of hydrazine. Neither hydroxylamine nor hydrazine have been isolated as intermediates. Accordingly, it was suggested[34] in the postulated case of hydrazine that attachment to the enzyme surface must be maintained until complete reduction to ammonia occurs. A very unusual binding site would be necessary since an increase in N–N bond distance from 1·09 Å for nitrogen to 1·47 Å for hydrazine must be taken into account. A hemoglobin-type site would have certain attractive possibilities in this regard since a number of gases and ions are reversibly complexed by the iron-porphyrin ring when in combination with an appropriate protein. In addition, the central iron in the heme is capable of reversible valency change with the somewhat unique result that the proteins will not maintain a complex with any known gas when the iron is in the trivalent state but will form complexes with a number of anions while in this oxidized state.[7] This ability to complex with and thus stabilize a reactive anion could be very significant.

The mechanism suggested by Hoch *et al.* appears to be reasonable and to be the only suggested mechanism which is consistent with all known facts regarding nitrogen fixation, but it does not account for the presence or possible function of legoglobin. It is implied that legoglobin is not the enzyme responsible for nitrogen fixation because each legoglobin component has only one metal group to act as an electron donor. Yet the valency change of legoglobin reported by Hamilton *et al.*[15] and the more recent demonstration of a nitrogen-legoglobin complex by Abel *et al.*[10] strongly suggests a key role for legoglobin. This investigator maintains that the mechanism suggested by Hoch is consistent with the view that legoglobin is the site of nitrogen fixation if certain reasonable assumptions are made. These assumptions are: (a) that a complex exists *in vivo* between the two legoglobin components, and (b) that a configurational change occurs in this complex as protons in the surrounding media (or within the legoglobin molecules) are converted to hydrogen.

HYPOTHESIS

Experimental Background

No complexing between the two legoglobin components has been demonstrated. However, it is well known that hemoglobin will dissociate into two molecules, each containing two heme groups.[35] The combination of spherical and assymmetric globular molecules[6] could provide many amino acid side chains across which hydrogen bonds could form. One requirement of this complex is that it forms in such a manner that the two heme groups are brought into close proximity to each other, thus essentially acting as a "cage" for the nitrogen molecules.

If such a legoglobin-to-legoglobin complex does exist *in vivo*, it may be extremely difficult to determine. The Arrhenius activation energy changes with the ammonium sulfate

[33] M. K. Bach, *Biochim. Biophys. Acta* **26**, 104 (1957).
[34] G. E. Hoch, K. C. Schneider and R. H. Burris, *Biochim. Biophys. Acta* **37**, 273 (1960).
[35] J. F. Taylor, *J. Biol. Chem.* **128**, 102 (1939).

concentration[10] and this is indicative of the breaking of hydrogen bonds. Accordingly, any fractionation procedure using ammonium sulfate would be expected to disrupt a hydrogen bonded complex and since the legoglobin molecule can undergo irreversible configurational changes even at $-10°$[10] it may not be possible to reform the complex for examination *in vitro*.

The specific globin configuration affects the properties of the porphyrin significantly. For example: in myoglobin and hemoglobin, a very low partial pressure of carbon monoxide in a high oxygen partial pressure will quickly bring about the replacement of the heme-complexed oxygen by carbon monoxide to form the more stable carboxyhemoglobins. In contrast to this, Davenport[24,25] has demonstrated that heme proteins isolated from certain parasitic organisms behave in the opposite manner; i.e., a low partial pressure of oxygen in a high carbon monoxide partial pressure will replace the carbon monoxide complex with oxygen to form an exceptionally stable oxyhemoglobin. Since the heme group is the same in each type of hemoglobin, it is obvious that the difference in properties is a function of the supporting globular protein. Only recently Wang *et al.*[36,37] have shown that the globin portion of the molecule definitely is responsible for the fact that oxygen can combine reversibly without resulting in oxidation of the divalent iron of hemoglobin. This knowledge combined with the recent work by Kendrew *et al.*[38] and Perutz *et al.*[39] on the three-dimensional structures of myoglobin and hemoglobin has provided a better insight into the possible reaction mechanisms of these complex molecules. It is now apparent that relatively minor configurational changes will affect significantly the characteristics of these proteins,[1,10,40] and it is conceivable that a small difference in configuration (and resultant exposure of specific amino acid groups) accounts for the surprising fact that carbon monoxide will normally form strong complexes with heme proteins while nitrogen, which is the same size, has the same bond lengths, and has the same electronic configuration, does not form complexes with any heme protein except legoglobin.

Bauer[30] has indicated that thermodynamically the evolution of hydrogen could be expected to occur at or near the same site as nitrogen attachment in an enzyme that has properties similar to legoglobin. If protons from the semi-isolated regions containing legoglobin were converted to hydrogen, it would be equivalent to changing the pH in such a diffusion controlled system, and a pH change does significantly affect the binding characteristics and stability of ferrolegoglobin.[10] The total absence of sulfur-containing amino acids and the low histidine content of both legoglobin components mentioned earlier further suggests that the three-dimensional configuration of each component is maintained by hydrogen bonding rather than the more rigid cross linked characteristics of the S–S bond. Accordingly, by assuming that a legoglobin-to-legoglobin complex exists and that a change in configuration occurs as hydrogen is produced and as nitrogen is reduced to ammonia, the following first step for the nitrogen fixation process is suggested.

Postulated Mechanism

Nitrogen, after diffusing as a water solution into the nitrogen-fixing cells is bound by the postulated ferrolegoglobin–ferrolegoglobin complex (henceforth referred to as the "ferro-

[36] H. H. WANG, A. NAKAHARA and E. B. FLEISCHER, *J. Am. Chem. Soc.* **80**, 1109 (1958).
[37] J. H. WANG, *J. Am. Chem. Soc.* **80**, 3168 (1958).
[38] J. C. KENDREW, R. E. DICKERSON, B. E. STRANDBORG, R. G. HART, D. R. DAVIES, D. C. PHILLIPS and V. C. SHORE, *Nature* **185**, 422 (1960).
[39] M. F. PERUTZ, M. G. ROSSMANN, A. F. COLLIS, H. MUIRHEAD, G. WILL and C. T. NORTH, *Nature* **185**, 416 (1960).
[40] K. ABEL, *Arch. Biochem. Biophysics*, In Press (1963).

enzyme"). Under the influence of the strong electrostatic field developed between the charged iron-porphyrin of each legoglobin component, the nitrogen molecule gains two electrons, one from each iron. The resulting ferri-enzyme would be capable of maintaining the $^{-}NN^{-}$ anion as a stable complex long enough for hydrogen, present in the surrounding media, to react with the ferri-enzyme to produce the ferro-enzyme and two protons which immediately would react with the nitrogen anion to yield the diimide, HNNH. Not only is the diimide molecule very unstable but the bond length is longer than in molecular nitrogen. In order for an adequate stabilizing complex to form between the ferro-enzyme and the diimide, a configuration change in the enzyme would be desirable. Since a change in pH does produce a configurational change [10] it is visualized that an unidentified reactive group in the immediate vicinity of the heme groups reacts with free protons (the nodule interior being acidic [30]) to produce hydrogen. In a semi-isolated system where diffusion is a controlling mechanism, this conversion of protons to hydrogen would result in an adequate pH change (with the media becoming less acidic) to produce a suitable configurational change. (The stability of the nitrogen complex increases as the acidity decreases [1, 10] which is consistent with the view that a stronger complex would be required to maintain the diimide than to hold the nitrogen molecule.)

The second step—conversion of the diimide to hydrazine—would proceed in an essentially identical manner with a further production of hydrogen and a further configurational change in the enzyme. The third step—conversion of hydrazine to ammonia—would also proceed in a like manner except that a pH dependent enzyme configurational change would not result in the binding of ammonia to the enzyme and these two resulting small molecules would be free to escape from the legoglobin "cage" and into a region where ammonia assimilation could occur. The final step of the cycle, returning the enzyme to the necessary configuration for assimilation of nitrogen and its conversion to $^{-}NN^{-}$, would be controlled by the rate of diffusion of protons into the system and re-establishment of the optimum pH to re-open the cage. Another nitrogen molecule can then complex provided molecules which will also complex with heme proteins (such as oxygen, carbon monoxide, or nitrous oxide) are not in competition for the heme site.

Discussion

This proposed pathway suggests a diffusion controlled system in which all conditions, including nitrogen-to-nitrogen bond lengths and enzyme configuration, must be optimum before fixation can occur. Certain further assumptions regarding such a system appear reasonable and would help to explain certain experimental findings. For example, it is reasonable to assume on the above basis that if nitrogen is not supplied to the nodules at an adequate rate to prevent a build-up of hydrogen gas above that which is necessary to maintain the legoglobin in the ferro state while providing readily available protons for assimilation by the nitrogen anions, hydrogen gas losses from the cells by diffusion will result in the additional conversion of protons to hydrogen by the legoglobin (with resulting configurational changes) so that nitrogen is no longer preferentially bound. If this occurred and the legoglobin extracted from the nodules, it would exhibit somewhat different properties than if it were extracted during active fixation. Furthermore, if a relatively large excess of hydrogen were supplied to the nodules, the conversion of protons to hydrogen would be inhibited according to the mass action laws controlling such reactions, thus preventing configuration changes in the legoglobin. As a direct result, nitrogen fixation would be inhibited because the reaction could not proceed past the formation of the diimide. A secondary result would be that

legoglobin extracted from nodules maintained in a high hydrogen concentration would be less likely to autoxidize than would legoglobin not extracted from hydrogen-rich nodules because the heme groups would still be located within the closed "cage". The results of Hamilton *et al.* and Abel *et al.* can be explained on this basis.

Although the above nitrogen fixation pathway may be subsequently shown to be inaccurate, significant evidence is now available to indicate that legoglobin is the enzyme responsible for symbiotic nitrogen fixation and that this fixation process is a reductive rather than an oxidative conversion of nitrogen.

Acknowledgements—The author is grateful for the encouragement and help given by Dr. N. Bauer (deceased) and Dr. J. Spence, as well as the support provided by the Herman Frasch Foundation and the Utah Agricultural Experiment Station.