Secondary gasation of heme proteins and biological N_2-fixation

Elsewhere[1] we have presented data on the solubility of N_2, H_2, and of A in aqueous legoglobin from soybean-root nodules, as well as in aqueous horse myoglobin and in horse hemoglobin. Here we wish to discuss the implications of these data and related information with respect to the problem of the initial steps in symbiotic N_2-fixation.

It has been established, mainly by VIRTANEN[2], that the capacity of leguminous root nodules to fix atmospheric N_2 goes roughly parallel to the concentration of the heme proteins found in the nodules. We prefer the term legoglobins for these heme proteins rather than leghemoglobin[3] since it is more consistent with the present terminology for the quite similar protein myoglobin. HAMILTON, SHUG AND WILSON[4] have reported an apparent oxidation of ferrous heme iron in root nodule extracts as a result of passing N_2 gas into the extract. These observations imply a possible interaction between N_2 and legoglobins, but are not conclusive in part for reasons discussed below.

We have found a weak interaction between N_2 and Lb, as well as one for Mb and for Hb, in harmony with the earlier discovery of VAN SLYKE *et al.*[5] that oxygenated Hb takes up N_2 to a small extent at 1 atm. The present results may be summarized by stating that the standard free energy of N_2 gas uptake by the heme proteins, $\Delta F°_u$, at 293°K lies within the narrow range of $+2.0_0$ to $+2.7_0$ kcal/mole, depending on the protein and its oxidation state. This means that at an N_2 pressure of 1 atm only about 1 % of the heme sites are occupied, if we assume one N_2 per heme. Such a weak interaction will be called secondary gasation, partly because it occurs whether primary gasation (*e.g.* oxygenation) has taken place or not. In spite of its weakness, though, secondary gasation seems to be specifically associated with the heme group or possibly with a metal cation, since the non-heme, non-metallic proteins investigated[5,6] so far show no affinity for N_2.

If we suppose that the first step in biological fixation of the N_2 molecule is its attachment to a specific reaction site on some other molecule "X", we must conclude that legoglobin is particularly well suited to play the role of "X". The standard free energy barrier for uptake of N_2 on $LbFe^{+2}$ (2.0_0 kcal/mole) is small relative to other probable barriers[7] in the eventual reduction of N_2 to NH_3; and is quite small relative to the limit of about 20 kcal/mole which reaction-rate theory places on feasible elementary steps of thermal reactions at room temperature. Because of the noble-gas character of N_2, it is difficult to conceive of any aqueous molecule "X" that would have more than a few percent of its sites occupied by N_2 at room temperature and pressure. Hence the total number of available sites per molecule of "X" must be large or the concentration of "X" must be larger than that of enzymes in usual biological systems. We estimate from our yields in the work cited above that the concentration of Lb in root nodules is about $1 \cdot 10^{-4}$ *M* at the height of N_2-fixing capacity, *i.e.* $1 \cdot 10^{-6}$ *M* in N_2-occupied sites at 1 atm. This is a remarkably high value, made possible by the relatively low molecular weight (20,000) and the high solubility of legoglobins. Local concentrations of Lb within the root cell-bacterial complex are likely to be even higher.

Legoglobin, with its Fe^{+2} (or Fe^{+3}) group, also offers the possibility of furnishing

Abbreviations: Mb, myoglobin; Hb, hemoglobin; Lb, legoglobin.

a reduction (or oxidation) potential just at the point where attachment of N_2 probably occurs, analogous to the probable action of cytochrome a_3 on the O_2 molecule. The redox properties of legoglobin are under investigation in this laboratory. It is interesting that the reduced state of Lb, $LbFe^{+2}$, has a somewhat greater N_2 affinity than the oxidized state, $LbFe^{+3}$, a situation more favorable for the reduction of N_2 in the presence of $LbFe^{+2}$ than for its oxidation in the presence of $LbFe^{+3}$. Oxidation affects myoglobin in a similar way, in harmony with other similarities; but with hemoglobin, the relative affinities of oxidized and reduced states are reversed.

It may be wondered if legoglobin alone is capable of reducing N_2. In our gas-desorption measurements we occasionally observed some slow conversion of $LbFe^{+2}$ to $LbFe^{+3}$ in the presence of N_2, reminiscent of the experiment by HAMILTON, SHUG AND WILSON[4]. However, the same behavior also was (non-reproducibly) observed in the presence of A and of H_2. This leads us to think that, in the absence of O_2 (< 30 p.p.m.) ferrolegoglobin is capable of a disproportionation reaction of the type hinted at by VIRTANEN *et al.*[2], obscuring a possible parallel reaction with N_2. Ascorbic acid prevented the above conversion; and it is noteworthy that ascorbic acid is abundant in root nodules[8]. However, ascorbic acid also lowered somewhat the apparent affinity of $LbFe^{+2}$ for N_2 ($\Delta F^\circ_u = +2.4_2$ kcal/mole), an effect that might be associated with errors in ΔF°_u due to traces of O_2 which ascorbic acid would clean up.

The above facts make it reasonable to hypothesize that a secondary attachment of N_2 to the heme group in legoglobin is the first step in symbiotic N_2-fixation.

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Photo-oxidation of yeast enolase

Kinetic studies have suggested that histidine residues are involved in both the catalytic action and the metal binding of yeast enolase[1–3]. In order to obtain further evidence for the participation of histidine in the activity of this enzyme, we have subjected it to photo-oxidation in the presence of methylene blue, and attempted