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

to determine quantitatively the changes in the protein which are associated with the loss of enzymic activity during the oxidation.

A reaction mixture consisting of 4.0 mg/ml yeast enolase and 0.06 mg/ml methylene blue in 0.05 *M* potassium phosphate buffer, pH 7.1, was irradiated with a 500-W light bulb at a distance of about 10 cm. The samples were maintained at 25–30° in a water bath. At several times aliquots were removed, assayed for activity as described previously[3], and freed of methylene blue by treatment with charcoal. The tryptophan content of the samples was determined colorimetrically[4] on the intact protein. The samples were then hydrolyzed in 6 *N* HCl, and the histidine and tyrosine contents were determined chemically[5,6] and by microbiological assay. Methionine was determined by microbiological assay.

The results of one experiment are summarized in Table I. Only histidine and tryptophan are oxidized to a significant extent during the inactivation of the enzyme. Since the only amino acids which are rapidly destroyed by methylene blue photo-oxidation are histidine, tryptophan, tyrosine, methionine, and cysteine[7], and since enolase contains no cysteine[8], the loss in activity in this case must be due to the destruction of histidine and/or tryptophan residues.

TABLE I

AMINO ACID CONTENT OF PHOTO-OXIDIZED ENOLASE

The values for the amino acid content of the native enzyme are based on data of MALMSTRÖM *et al.*[5].

Oxidation time (min)	*Activity (%)*	*Histidine (moles/mole enolase)*	*Tyrosine (moles/mole enolase)*	*Tryptophan (moles/mole enolase)*	*Methionine (moles/mole enolase)*
0	100	14.0	15.0	5.0	8.0
5	87	14.0	15.4	4.9	9.0
20	58	12.0	13.2	4.5	5.4
40	13	9.5	14.7	3.8	7.4
58	1	6.3	15.3	2.5	7.9

TABLE II

ANALYSIS OF PHOTO-OXIDIZED ENOLASE IN THE ULTRACENTRIFUGE

Activity (%)	*Protein concentration* (mg/ml)*	S_{20} *(sec × 10¹³)*	*Apparent diffusion coefficient at 20° (cm²/sec × 10⁷)*
100**	1.8	5.68	1.51
60	1.5		1.37
27	1.8	6.32	1.57

* Determined spectrophotometrically[9].

** The sample was subjected to exactly the same treatment as the oxidized protein with the exception that methylene blue was omitted.

The oxidized enolase was subjected to physical tests to determine whether photo-oxidation affected the tertiary structure of the enzyme. Although viscosity measurements conducted under suboptimal conditions of protein concentration and flow time showed no significant change over a range of 0 to 90 % inactivation, sedimentation measurements gave evidence of structural changes during photooxidation.

Prior to measurement of the sedimentation coefficient in the Spinco Model E Ultracentrifuge, the samples were dialyzed against several changes of 1 M KCl in 0.05 M potassium phosphate buffer, pH 7.0, to remove the methylene blue, and then dialyzed against buffer alone to remove the KCl.

The results of the ultracentrifuge experiments are collected in Table II. The apparent diffusion coefficient was calculated from the ultracentrifuge patterns according to the equation[10]

$$D_{app} = \frac{1}{4\pi t}\left(\frac{A}{B}\right)^2$$

(The term correcting for the effect of the centrifugal field on different parts of the boundary ($1 - \omega^2 st$) was found to be insignificant and was thus omitted.) We used D_{app} as a measure of the broadening of the Schlieren image of the boundary and interpreted the boundary spreading as a crude measure of heterogeneity of the protein sample.

Photo-oxidation of yeast enolase thus causes complete loss of activity after the destruction of 8 histidine residues and 2–3 tryptophan residues. This rather extensive oxidation is accompanied by significant changes in the structure of the protein as indicated by the increase in both the sedimentation coefficient and the apparent diffusion coefficient.

Only in instances where the chemical modification of a single group in the enzyme leads to complete loss of activity without altering other parameters is there justification for implicating the modified group in the active site of the enzyme. The data obtained in this study are clearly not subject to this kind of interpretation and earlier conclusions as to the involvement of histidine in the active site of enolase[3] should be re-evaluated.

MALMSTRÖM has recently proposed that 12 of the 14 histidine residues in enolase are involved in the tertiary structure of the native protein[8]. Our findings possibly lend some support to this proposal, since the oxidation of 8 histidine groups involved in such bonding might well lead to inactivation of the enzyme.

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