Biochimica et Biophysica Acta, 614 (1980) 303—312

BBA 69040

PEROXIDASE CONTENT OF SOYBEAN ROOT NODULES

A. PUPPO [a], J. RIGAUD [a], D. JOB [b], J. RICARD [b] and B. ZEBA [b]

[a] *Laboratoire de Biologie végétale, Faculté des Sciences et des Techniques, 06034 Nice Cedex and* [b] *Laboratoire de Physiologie cellulaire végétale associé au C.N.R.S., U.E.R. Scientifique de Luminy, 13288 Marseille Cedex 2 (France)*

(Received October 10th, 1979)
(Revised manuscript received March 4th, 1980)

Key words: Leghemoglobin; Peroxidase; (Soybean root nodule)

Summary

A peroxidase has been isolated from soybean nodules and its main characteristics have been determined. Its molecular weight (48 000) and spectral properties are similar to those of usual plant peroxidases. Its activity is comparable to that of low-efficiency plant peroxidases. The rate constant of the reaction with H_2O_2 is $3 \cdot 10^5\ M^{-1} \cdot s^{-1}$. In this reaction, nodule peroxidase yields an oxidized intermediate analogous to the compound I species of peroxidases already studied. A comparison is made with the pseudoperoxidatic activity of soybean leghemoglobin components. Leghemoglobins *a* and *c* react with H_2O_2 with rate constants of $5 \cdot 10^3$ and $2.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$, respectively, yielding the leghemoglobin (IV) species. During these reactions leghemoglobins are inactivated.

Introduction

Soybean root nodules (*Glycine max*) contain red pigments, leghemoglobins, which are monomeric hemoproteins with molecular weight of about 16 000 [1]. In the root nodules, they exist mostly in the reduced form [2] and their main biological function seems to act by facilitating the diffusion and delivery of oxygen to the bacteroids [3]. Besides this important function, pseudoperoxidatic activity [4] and indole-3-acetic acid oxidase activity [5] of the ferric form of these hemoproteins have been reported. This pseudoperoxidatic activity has been commonly used to determine the cellular location of leghemoglobins [6,7]. Since this function is solely the property of the ferric form [4,5], it has been suggested that it might become important during nodule aging, when leghemoglobin is oxidized [8]. Oxidation of aromatic substrates by H_2O_2 and oxidation of indole-3-acetic acid are usually catalyzed, in plant cells,

by peroxidases [9,10]. The finding that root nodules of several leguminous plants contain a great number of peroxidase isoenzymes [11] has recently been questioned by Sievers and Rönnberg [4]. Nevertheless, a typical peroxidase activity which cannot be confused with leghemoglobins has been detected in soybean root nodules by Puppo and Rigaud [5]. The aim of this study is to evaluate the peroxidatic content of soybean root nodules, and to compare the activity of the already detected nodule peroxidase [5] with that of leghemoglobins.

Materials and Methods

Soybean (*Glycine max* Merr. cv. Altona) nodules were obtained as described previously [5]. Leghemoglobin purification procedures are indicated elsewhere [5]. The elution profile of a crude preparation of soybean leghemoglobin on an 80 × 2.5 cm column of Sephadex G-100 showed the presence of two peaks of peroxidase activity: the first corresponding to the nodule peroxidase [5] and the second to leghemoglobin (mixture of leghemoglobins *a* and *c*). H_2O_2, guaiacol and $KFe(CN)_6$ were from Merck. H_2O_2 concentration was determined just before experiments using the iodide assay [12]. Absorbance measurements were effected with a Beckman Acta M VII spectrophotometer maintained at 25°C. Molecular weight determination of nodule peroxidase was effected by molecular sieving. A Sephadex G-100 column (80 × 2.5 cm) was equilibrated with 0.1 M phosphate buffer, pH 6.8, containing 0.5 M KNO_3. Standard proteins (bovine serum albumin, ovalbumin, ribonuclease and α-chymotrypsinogen) as well as nodule peroxidase were dissolved in this buffer and applied to the column and fractions of 1.5 ml were collected. The flow rate was 7.5 ml/h. Gel filtration experiments were run at 4°C. Protein concentration of the fractions was determined spectrophotometrically at 280 nm for the protein standards, and at 403 nm for peroxidase. The molecular weight of peroxidase was estimated from its elution pattern relative to the known standards.

Peroxidase activities were measured with guaiacol as the electron donor, using a molar absorptivity of $5.6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ for the oxidation product, at 470 nm, per mol of guaiacol oxidized [13]. The manner of conducting both the experiments and the analysis of the data is similar to that described by Santimone [13] for the reaction of guaiacol oxidation by H_2O_2, catalyzed by horseradish peroxidase, under steady-state conditions. This approach has also been used for the study of substrate electron donor oxidation by H_2O_2, catalyzed by lactoperoxidase [14]. Doubly distilled water was used to prepare all solutions. All reactions were performed at pH 6.8 in a 0.1 M phosphate buffer.

Results

Characterization of peroxidase activities in soybean nodules

From the protein and heme contents in the two peaks of peroxidase activity [5], one can estimate that there is an approximately 50-fold excess of leghemoglobin over peroxidase. Electrophoresis on cellulose acetate of the peroxidase revealed that the enzyme is negatively charged at pH 5.2 (12 mM acetate

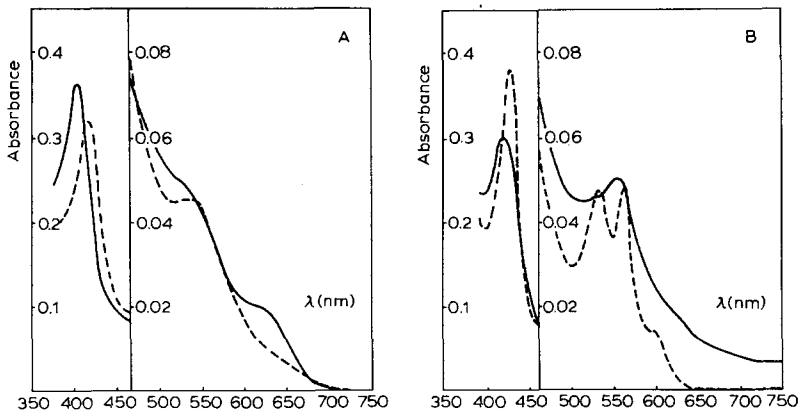


Fig. 1. A. Absorption spectra of 3.7 μM ferric nodule peroxidase (———) and its cyanide complex (- - - - -). B. Absorption spectra of 3.7 μM ferrous nodule peroxidase (———) and its cyanide complex (- - - - -). The pH is 6.8 (0.1 M phosphate buffer).

buffer). Its molecular weight is 48 000. The absorption spectra of the ferric and ferrous forms of nodule peroxidase, together with those of their complexes with cyanide are presented in Fig. 1. Heme titration compared with the absorption in the Soret region (403 nm) gave rise to an ϵ_{403} of $10^5\ M^{-1} \cdot cm^{-1}$ for ferric nodule peroxidase, in good agreement with the values encountered for peroxidases already studied [9]. The absorption spectrum of the ferrous form, at alkaline pH and in the presence of pyridine, was characteristic of protoporphyrin IX [15]. No spectral change was obtained after mixing either the ferric or the ferrous form of the peroxidase with nicotinic acid.

Peroxidase activity of nodule peroxidase and leghemoglobins

The peroxidase is present in only very small amounts in nodule extracts. Its catalytic activity was thus appreciated by the measure of guaiacol oxidation by H_2O_2, under steady-state conditions. Rate measurements were effected at various guaiacol and H_2O_2 concentrations. The same measurements were effected with leghemoglobins *a* and *c*. Different results were obtained, depending on the order of addition of hydrogen peroxide and guaiacol to leghemoglobins. Higher velocities were measured when guaiacol was introduced first, followed by H_2O_2, in reaction cuvettes containing leghemoglobin solutions. This order of addition of substrates was used throughout in the study of guaiacol oxidation by leghemoglobins. This kinetic problem was not encountered for reactions catalyzed by nodule peroxidase. The results are presented in Fig. 2, in the form of double-reciprocal plots, $[E]_0/v$ vs. 1/[guaiacol], where $[E]_0$ stands for peroxidase or leghemoglobin concentration and v for the steady-state rate of guaiacol oxidation product appearance. In contrast to the results obtained for one H_2O_2 concentration by Sievers and Rönnberg [4], these plots are not linear, probably because the guaiacol concentration range studied here is larger than that mentioned in Ref. 4. The curvature of the plots is probably due to inhibition of the three ferric hemoproteins by excess guaiacol.

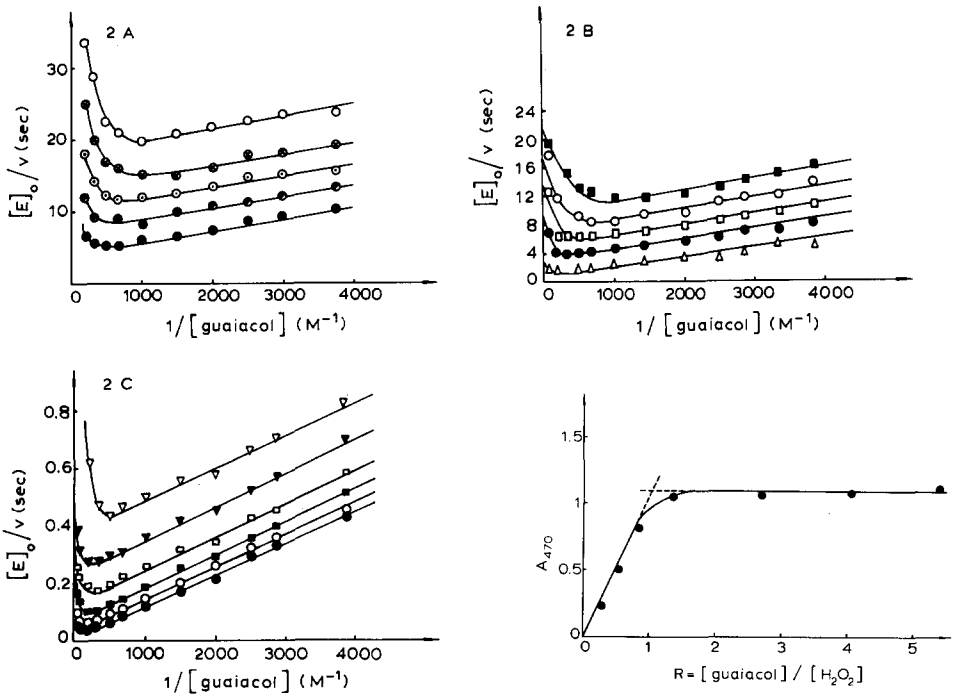


Fig. 2. Oxidation of guaiacol by leghemoglobin *a* (A), leghemoglobin *c* (B) and nodule peroxidase (C). The reactions were measured at 470 nm, in a 0.1 M phosphate buffer pH 6.8; temperature, 25° C. Hemoprotein concentrations, $[E]_0$, were: leghemoglobin *a*, 0.16 μM, leghemoglobin *c*, 0.08 μM and nodule peroxidase, 9 nM. Steady-state rates, *v*, were measured at various guaiacol and H_2O_2 concentrations. A. H_2O_2 concentrations are: 14.5 μM (○), 18 μM (○), 22 μM (⊙), 32 μM (⊘) and 65 μM (●). B. H_2O_2 concentrations are: 44 μM (■), 74 μM (○), 148 μM (□), 296 μM (●) and 1.19 mM (△). C. H_2O_2 concentrations are: 9 μM (▽), 15 μM (▼), 18.1 μM (□), 38 μM (■), 75.4 μM (○) and 136 μM (●).

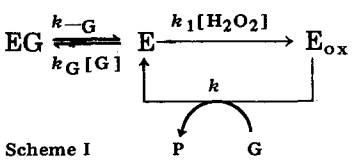
Fig. 3. (Bottom, right). Titration of hydrogen peroxide by guaiacol in the presence of leghemoglobin a. Absorbance changes are plotted against the ratio [guaiacol]/[H_2O_2]. The concentrations of leghemoglobin a and H_2O_2 are 0.175 μM and 217 μM, respectively. The pH is 6.8 (0.1 M phosphate buffer). Exactly the same results are obtained in the presence of leghemoglobin c and nodule peroxidase.

An example of titration of hydrogen peroxide by guaiacol, with a catalytic concentration of ferric leghemoglobin *a* is shown in Fig. 3. The results can be interpreted by a 1 : 1 stoichiometry in the overall process. Exactly the same results were obtained using leghemoglobin *c* and nodule peroxidase.

The effect of nicotinic acid was tested on the peroxidase activity of leghemoglobin *c*. This ligand was found to inhibit competitively the reaction of the hemoprotein with H_2O_2.

Discussion

A minimum model which would account for the above results is Scheme 1:



Scheme I

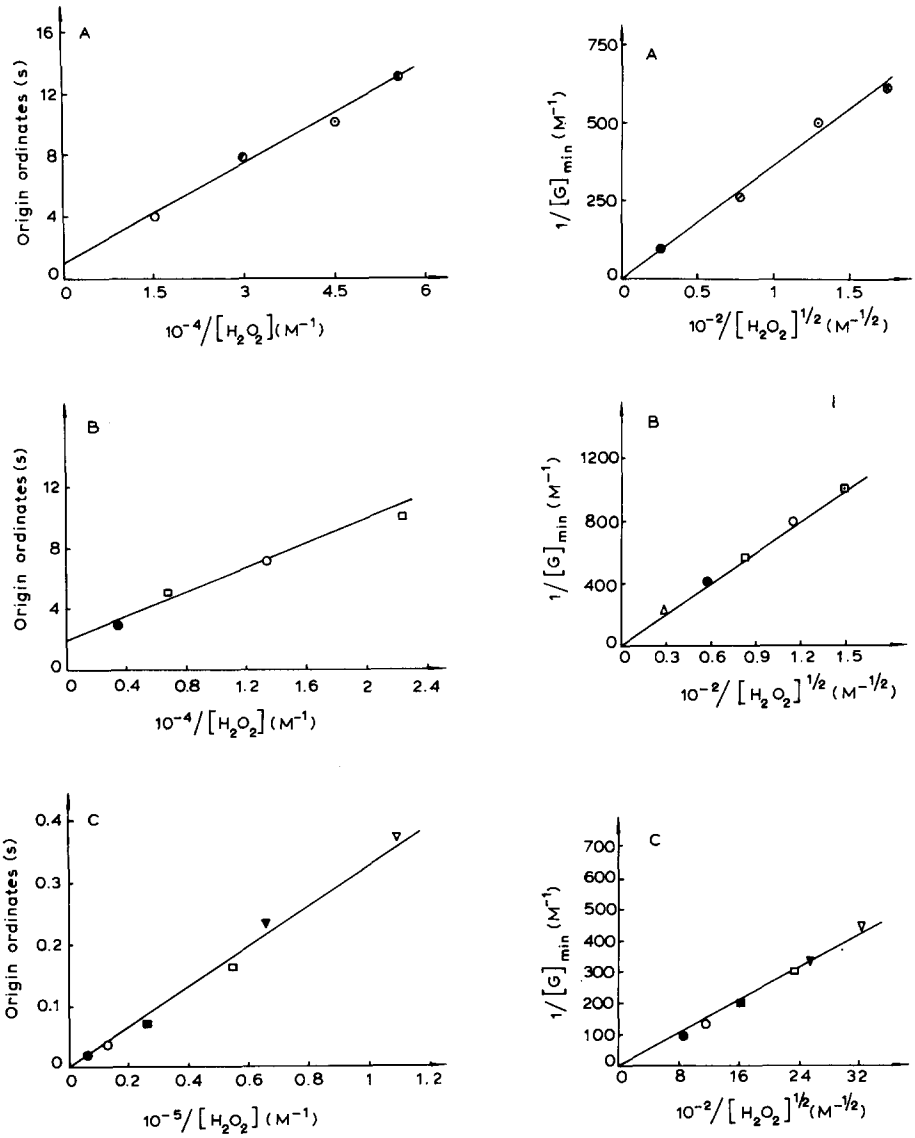


Fig. 4. Secondary plots deduced from Lineweaver-Burk plots of Fig. 2, and corresponding to leghemoglobin *a* (A), leghemoglobin *c* (B), and nodule peroxidase (C). The origine ordinates of the linear portions of the curves in Fig. 2 (low guaiacol concentration range) are plotted against $[H_2O_2]^{-1}$. The data are those of Fig. 2. Straight lines correspond to Eqns. 3 and 6 in the text.

Fig. 5. Secondary plots deduced from Lineweaver-Burk plots of Fig. 2, corresponding to leghemoglobin *a* (A), leghemoglobin *c* (B) and nodule peroxidase (C). The abscissa of the minimum ($1/[G]_{min}$) in the plots of Fig. 2 are plotted against $[H_2O_2]^{-1/2}$. The data are those of Fig. 2. Straight lines correspond to Eqn. 7 in the text.

in which G and P represent guaiacol and its oxidation product, respectively; E represents the ferric hemoprotein (leghemoglobin *a* or *c*, or peroxidase) and E_{ox} its oxidized compound. In the case of leghemoglobins, the reaction between the ferric form and H_2O_2 yields an oxidized leghemoglobin form

[4,16,17] called leghemoglobin (IV). In Scheme I, EG is an abortive complex between ferric hemoprotein and guaiacol, and is introduced to account for the curvature of the plots in Fig. 2. Applying the steady-state approximation to the enzyme species in Scheme I allows us to express the rate, v, of appearance of product P by Eqn. 1:

$$\frac{[E]_0}{v} = \frac{1}{k[G]} + \frac{1}{k_1[H_2O_2]} + \frac{K_G[G]}{k_1[H_2O_2]} \quad (1)$$

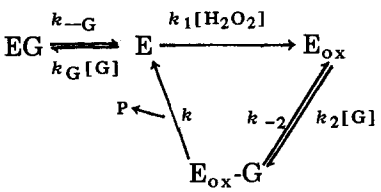
where $K_G = k_G/k_{-G}$. Eqn. 1, deduced from Scheme I, appears to account for the experimental results in Fig. 2. Thus, for low guaiacol concentrations, the $[E]_0/v$ vs. $1/[\text{guaiacol}]$ plots should be linear, with identical slope $1/k$:

$$\left(\frac{[E]_0}{v}\right)_{[G]\to 0} = \frac{1}{k[G]} + \frac{1}{k_1[H_2O_2]} \quad (2)$$

For high guaiacol concentrations, the plots are no longer linear, because of the presence of the last term in Eqn. 1. From Eqn. 2, one can deduce that the linear portions of the curves in Fig. 2 have origin ordinates which depend on hydrogen peroxide concentration, according to Eqn. 3:

$$\text{Origin ordinates of the linear portions} = \frac{1}{k_1[H_2O_2]} \quad (3)$$

The plots corresponding to Eqn. 3 are shown in Fig. 4. Eqn. 3 predicts that these plots should have zero intercepts, a result which is observed only for nodule peroxidase (Fig. 4C). For both leghemoglobins a and c the intercepts differ significantly from zero (Fig. 4A and B). Model II is formally identical to model I, except that a step of formation of a binary complex between oxidized hemoprotein and guaiacol precedes the limiting step of product release:



Scheme II

Under steady state approximation, Scheme II leads to Eqn. 4:

$$\frac{[E]_0}{v} = \frac{1}{k} + \frac{1}{k_1[H_2O_2]} + \frac{(k + k_{-2})}{k \cdot k_2} \cdot \frac{1}{[G]} + \frac{K_G[G]}{k_1[H_2O_2]} \quad (4)$$

For low guaiacol concentrations, the plots $[E]_0/v$ vs. $1/[\text{guaiacol}]$ are parallel straight lines, with slope $(k + k_{-2})/kk_2$:

$$\left(\frac{[E]_0}{v}\right)_{[G]\to 0} = \frac{1}{k} + \frac{1}{k_1[H_2O_2]} + \frac{(k + k_{-2})}{k \cdot k_2} \cdot \frac{1}{[G]} \quad (5)$$

The origin ordinates of the linear portions of the curves are related to H_2O_2 concentration by Eqn. 6:

$$\text{Origin ordinates of the linear portions} = \frac{1}{k} + \frac{1}{k_1[H_2O_2]} \quad (6)$$

TABLE I

GUAIACOL OXIDATION BY PEROXIDASE AND LEGHEMOGLOBINS *a* AND *c*

Values of the parameters corresponding to Scheme II. The experimental conditions are those of Fig. 2.

	Peroxidase	Leghemoglobin *a*	Leghemoglobin *c*
k_1 ($M^{-1} \cdot s^{-1}$)	$3.2 \cdot 10^5$	$4.7 \cdot 10^3$	$2.5 \cdot 10^3$
k (s^{-1})	$\geqslant 100$	1	0.6
K_G (M^{-1})	55	110	180

Therefore, the plots corresponding to Eqn. 6 do not have zero intercepts. Examination of the plots in Fig. 4 indicates that k is very large for the nodule peroxidase (at least 100 s^{-1}), in agreement with the finding that this hemoprotein has a much higher specific activity than leghemoglobins *a* and *c*. From the plots in Fig. 4, one can also deduce the values of k_1, rate constant for reaction of the hemoproteins with hydrogren peroxide. The k and k_1 values are assembled in Table I.

The double-reciprocal plots in Fig. 2 present a minimum for guaiacol concentration, $[G]_{min}$, which can be deduced from Eqn. 5 as:

$$\frac{1}{[G]_{min}} = \sqrt{\frac{K_G k k_2}{k_1(k + k_{-2})}} \cdot \frac{1}{\sqrt{H_2O_2}} \tag{7}$$

Therefore, a plot of the abscissa of the minimum ($1/[G]_{min}$) vs. $[H_2O_2]^{1/2}$ should be linear, with zero intercept. Fig. 5 shows that, for nodule peroxidase, leghemoglobins *a* and *c*, the results are consistent with Eqn. 7. From the value of the linear portions in Fig. 2, $(k + k_{-2})/kk_2$, the values of k_1 in Table I and the values of the slopes of the plots corresponding to Eqn. 7 (Fig. 5), the values of K_G can be calculated. They are also assembled in Table I.

The titration experiments shown in Fig. 3 can be interpreted by a 1 : 1 stoichiometry in the reaction of guaiacol oxidation by H_2O_2, with catalytic amounts of the three hemoproteins studied. Classical ferriheme hydroperoxidase enzymes react with hydrogen peroxide to yield the catalytic intermediate compound I [9,14,18,19]. These reactions are 2*e*-equivalent oxidation of the Fe(III) native enzymes and two molecules of an electron donor are needed to reduce 1 mol of compound I to ferriperoxidase. Since it was shown that guaiacol [20] or *p*-cresol [21] behave, depending on the experimental conditions such as pH, as one- or two-electron donors in reactions catalyzed by horseradish peroxidase, the 1 : 1 stoichiometry reported here cannot give any firm indication of the nature of the oxidized compound formed by reaction of the hemoproteins with H_2O_2. When ferrocyanide, instead of guaiacol, is used as the electron donor, in the reaction catalyzed by nodule peroxidase, the titration curve presented in Fig. 6 shows a change of slope for a 2 : 1 ratio of ferrocyanide to peroxide. These results clearly indicate that ferric nodule peroxidase reacts with H_2O_2 to yield an oxidized intermediate analogous to the compound I species of peroxidases already studied. Unfortunately, these experiments cannot be reproduced with leghemoglobins *a* and *c*, because they are

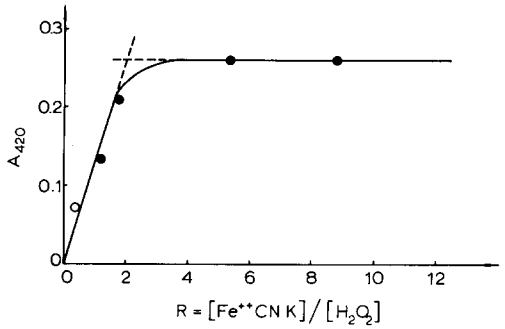


Fig. 6. Titration of hydrogen peroxide in the presence of nodule peroxidase. Absorbance changes at 420 nm (absorption maximum corresponding to ferricyanide) are plotted against the ratio [ferrocyanide]/[H_2O_2]. The concentrations of peroxidase and hydrogen peroxide are 7 nM and 148 μM, respectively. The pH is 6.8 (0.1 M phosphate buffer).

almost completely inactive in the ferrocyanide oxidation reaction by H_2O_2. Nevertheless, it is well established that ferric leghemoglobins react with H_2O_2 in a 1 : 1 molar ratio to form a peroxide compound which resembles compound II of hydroperoxidase enzymes [4], as does the reaction between myoglobin and H_2O_2 [22—24]. The product is one oxidation equivalent above ferric leghemoglobin or ferric myoglobin [17,22—24], the formal oxidation state accordingly is IV and the compounds have been called leghemoglobin (IV) and myoglobin (IV). Leghemoglobin (IV) can be formed either by reaction of ferric or ferrous leghemoglobins with H_2O_2 [17]. The second-order rate constant for reaction of ferrous leghemoglobin *a* or *c* with H_2O_2 is $2.3 \cdot 10^4\ M^{-1} \cdot s^{-1}$; that for the reaction of the ferric form is smaller [17], in accord with the results presented in Table I. Leghemoglobin (IV) is formed more quickly with leghemoglobin *a* than with leghemoglobin *c* [4], which is also in agreement with the results in Table I. According to Aviram et al. [17], during the reaction of leghemoglobin (IV) formation from ferric leghemoglobin, extensive side-reactions frequently occur, as noted for the reaction of ferric myoglobin with H_2O_2 [22]. In the latter reaction are generated transient $OH^{\cdot}$ radicals which may react destructively with aromatic residues of the protein [25] or with the heme group [22]. Evidence supporting inactivation of leghemoglobin during the reaction with hydrogen peroxide is given in Fig. 7. Formation of leghemoglobin (IV), from stoichiometric amounts of ferric leghemoglobin and H_2O_2 results in a decrease of absorbance at 403 nm [4]. If excess guaiacol is added to the preformed leghemoglobin (IV) species, the absorbance at 403 nm increases slightly, but the initial value is not reached, as would be expected if leghemoglobin (IV) was reduced by guaiacol back to ferric leghemoglobin (Fig. 7, curve 1). If, instead of adding guaiacol to the preformed complex, the guaiacol is first added to ferric leghemoglobin, and H_2O_2 is then introduced, leghemoglobin IV formation is observed at almost the same initial rate as previously. The absorbance at 403 nm then increases until the initial value is reached (curve 2): i.e., the ferric form has been regenerated. Experiments identical to those represented by curve 2 (Fig. 7) but followed at 470 nm (absorption maximum of the oxidation product of guaiacol) show that during the reaction the guaiacol is effectively

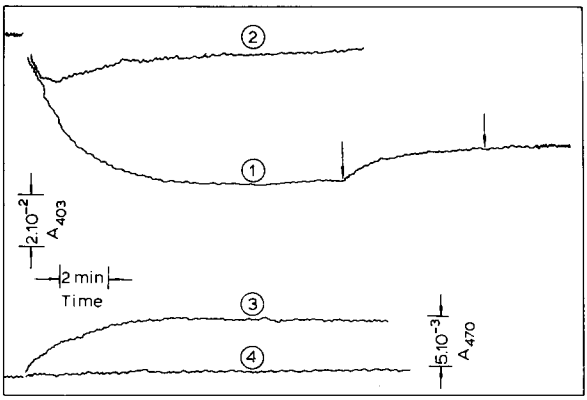


Fig. 7. Effect of guaiacol on the formation and decomposition of leghemoglobin (IV). A solution of leghemoglobin a 2.68 μM in 0.1 M phosphate buffer (pH 6.8) is mixed with H_2O_2 2.2 μM, in the absence (curves 1 and 4) or in the presence (curves 2 and 3) of 56 μM guaiacol. The reactions are followed at 403 nm (curves 1 and 2) and at 470 nm (curves 3 and 4). Arrows indicate successive additions of 56 μM guaiacol.

oxidized (curve 3). On this basis it appears that a normal peroxidase cycle, leghemoglobin (III) ⇌ leghemoglobin (IV), can be obtained only when the reducing agent is added to the ferric leghemoglobin before introducing H_2O_2. The results of Fig. 7 thus explain the effect of the order of addition of substrates to leghemoglobin solutions on the rate of appearance of guaiacol oxidation product. It should be noted that nicotinic acid, which exhibits a very high affinity for ferric leghemoglobins [26], inhibits leghemoglobin (IV) formation (Ref. 17 and this study). This ligand, which has been described as a natural regulator of leghemoglobin function [26,27] could perhaps provide an opportunity for preventing leghemoglobin inactivation by peroxides.

In the root nodules, leghemoglobins exist mostly in the reduced form [2]; therefore the concentration of the ferric form is probably very small and would commensurate with the concentration of the nodule peroxidase. Since specific activity of the nodule peroxidase is much higher than that of leghemoglobins (Table I and Fig. 2), the contribution of leghemoglobins in peroxidatic reactions, within the root nodules, is probably not significant compared to that of nodule peroxidase. The properties of the nodule peroxidase reported here are to be compared to those of low-efficiency plant peroxidases, such as horseradish peroxidase A_2 [19] or turnip peroxidase P_7 [18]. Thus, the rate constants for reaction of these hemoproteins with H_2O_2 are in the order of 10^6 $M^{-1} \cdot s^{-1}$, whereas those of high-efficiency plant peroxidases such as horseradish peroxidase C_2 [19] or turnip peroxidase P_1 are in the order of 10^7 $M^{-1} \cdot s^{-1}$. One may wonder why peroxidase activity in the nodule is slight. It should be noted that the oxygen tension inside the nodule is very low [28] and that the autoxidation process of oxyleghemoglobin, which would generate H_2O_2 via the spontaneous dismutation reaction of superoxide radicals, has been shown to be 2-times slower than that of myoglobin [29]. This indicates that appearance of H_2O_2 in the nodules is probably limited. In contrast with the results presented by Moustafa [11] only one peroxidase enzyme is predominant in nodule

extracts [5]. One should keep in mind that the method used to reveal the peroxidase isoenzymes [11], i.e. staining by the benzidine reagent in the presence of hydrogen peroxide, is not specific and can reveal all the hemoproteins present in nodule extracts. Apart from leghemoglobins a and c, soybean nodules also contain three other leghemoglobin components [30] and various hemoproteins [28].

References

1 Ellfolk, N. (1960) Acta Chem. Scand. 14, 1819—1827
2 Appleby, C.A. (1969) Biochim. Biophys. Acta 188, 222—229
3 Bergersen, F.J., Turner, G.L. and Appleby, C.A. (1973) Biochim. Biophys. Acta 292, 271—282
4 Sievers, G. and Rönnberg, M. (1978) Biochim. Biophys. Acta 533, 293—301
5 Puppo, A. and Rigaud, J. (1975) Physiol. Plant. 35, 181—185
6 Truchet, G. (1972) C.R. Acad. Sci. (Paris) Sér. D 274, 1290—1293
7 Bergersen, F.J. and Goodchild, D.J. (1973) Aust. J. Biol. Sci. 26, 741—756
8 Virtanen, A.I. and Laine, I. (1946) Nature 157, 25—29
9 Dunford, H.B. and Stillman, J.S. (1976) Coord. Chem. Rev. 19, 187—251
10 Ricard, J. and Job, D. (1974) Eur. J. Biochem. 44, 359—374
11 Moustafa, E. (1963) Nature 199, 1189
12 Cotton, M.L. and Dunford, H.B. (1973) Can. J. Chem. 51, 582—587
13 Santimone, M. (1973) Doctoral Thesis, University of Aix-Marseille, France
14 Maguire, R.J., Dunford, H.B. and Morrison, M. (1971) Can. J. Biochem. 49, 1165—1171
15 Falk, J.E. (1964) in Porphyrins and Metalloporphyrins, Elseiver Publishing Company, Amsterdam
16 Kubo, H. (1939) Acta Phytochim. 11, 195—200
17 Aviram, I., Wittenberg, B.A. and Wittenberg, J.B. (1978) J. Biol. Chem. 253, 5685—5689
18 Job, D., Ricard, J. and Dunford, H.B. (1978) Can. J. Biochem. 56, 702—707
19 Marklund, S., Ohlsson, P.I., Opara, A. and Paul, K.G. (1974) Biochim. Biophys. Acta 350, 304—313
20 Santimone, M. (1975) Can. J. Biochem. 53, 649—657
21 Hewson, W.D. and Dunford, H.B. (1976) J. Biol. Chem. 251, 6043—6052
22 George, P. and Irvine, D.H. (1952) Biochem. J. 52, 511—517
23 George, P. and Irvine, D.H. (1956) J. Colloid, Sci. 11, 327—339
24 Yonetani, T. and Schleyer, H. (1967) J. Biol. Chem. 242, 1974—1979
25 Weiss, J.J. (1964) Nature 202, 83—84
26 Appleby, C.A., Wittenberg, B.A. and Wittenberg, J.B. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 564—568
27 Appleby, C.A., Wittenberg, B.A. and Wittenberg, J.B. (1973) J. Biol. Chem. 248, 3183—3187
28 Appleby, C.A. (1974) in The Biology of Nitrogen Fixation, pp. 521—554, North-Holland Publishing Company, Amsterdam
29 Stetzkowski, F. and Banerjee, R. (1977) in Interactions Moléculaires de l'Hémoglobine, Vol. 70, pp. 107—112, INSERM
30 Appleby, C.A., Nicola, M.A., Hurrell, J.G.R. and Leach, S.J. (1974) Biochemistry 14, 4444—4450