Oxidation of Glycine by *Phaseolus* Leghaemoglobin with Associated Catabolic Reactions at the Haem

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(Received 9 February 1978)

Leghaemoglobin from the root nodules of kidney bean (*Phaseolus vulgaris*) reacts in alkaline glycine solutions as a glycine oxidase in a reaction that may also be regarded as a coupled oxidation. Leghaemoglobin is reduced to the ferrous form by glycinate, the oxygen complex is formed, and finally the haem is attacked to yield a green reaction product. Glycine is simultaneously oxidized to glyoxylate, and hydrogen peroxide is generated. The initial velocity of the formation of the green product is proportional to the concentrations of leghaemoglobin and glycine, and the optimum pH for the reaction is 10.2. The green product is not formed if carbon monoxide, azide or imidazole is bound to the haem, whereas oxidation of glycine to glyoxylate is not inhibited by azide and not essentially by carbon monoxide. Haem breakdown is activated by digestion of leghaemoglobin by carboxypeptidase, and partly inhibited by catalase and superoxide dismutase.

The haem group of mammalian haemoproteins is degraded enzymically *in vivo*, or it may be degraded chemically *in vitro* by so-called coupled oxidation with O₂ and ascorbate, the latter acting as a reductant (for reviews, see O'Carra, 1975; Schmid & McDonagh, 1975). These two apparently different reactions show considerable resemblance, but many details of each remain unresolved. In both reactions a methine carbon atom is hydroxylated and subsequently split off as carbon monoxide, yielding biliverdin, the end product of coupled oxidation. The route *in vivo* also involves further enzymic reduction to bilirubin.

The chemical degradation of the haem group of kidney-bean leghaemoglobin discussed in the present paper is not unrelated to the coupled oxidation of haemoproteins with O₂ and ascorbate, although the end products of the haem may be different. The reaction described here may assist understanding of the mechanisms involved in haem degradation.

The primary structure of a haemoprotein has been shown to have great significance in the stereoselectivity of the chemical degradation of the haem (O'Carra & Colleran, 1969, 1970; O'Carra, 1975), possibly by determining the tertiary structure near the haem group (Brown, 1976). The present paper shows that the haem group of kidney-bean leghaemoglobin is degraded by glycinate, whereas the haem group of soya-bean leghaemoglobin is not. The amino acid sequences of these proteins differ only by 21%, and most of the differences in their primary structures can be explained by conservative point mutations (Lehtovaara & Ellfolk, 1975).

The glycine oxidase activity of a leghaemoglobin is

especially surprising because the function of leghaemoglobins is, according to present knowledge, to facilitate O₂ diffusion within leguminous root nodules, to supply ATP for the bacterial nitrogenase (Wittenberg *et al.*, 1974; Bergersen & Turner, 1975). Such an O₂ carrier should of course not be susceptible to oxidative or reductive attack.

Glycine is also oxidized by amino acid oxidase and, interestingly, by ferric cytochrome *c* (Aviram, 1972). The reducing equivalents from glycine are in the enzymic reaction transferred to a flavin coenzyme, and in the cytochrome reaction to the ferric haem iron. Both reactions occur in a hydrophobic pocket formed by the protein. Although leghaemoglobin is totally different from cytochrome in both primary structure and function, it acts as a glycine oxidase in an apparently similar manner. The present paper is particularly concerned with this glycine oxidative reaction of kidney bean leghaemoglobin, whereas only the main points concerning the structure of the degraded haem are reported.

Experimental

Materials

Proteins. The leghaemoglobins from the root nodules of kidney bean (*Phaseolus vulgaris* var. Kaiser Wilhelm) and soya bean (*Glycine max*) were purified as described previously (Lehtovaara, 1977), and purity was verified by discontinuous gel electrophoresis. Sperm-whale myoglobin, human haemoglobin, carboxypeptidase A treated with diisopropyl phosphorofluoridate (EC 3.4.12.2, 55 units/mg of protein), catalase (EC 1.11.1.6, 3400 units/mg

of protein) and superoxide dismutase (EC 1.15.1.1, 2700 units/mg of protein) were all from Sigma Chemical Co., St. Louis, MO, U.S.A. One unit of carboxypeptidase A and catalase activity was the amount of enzyme required to catalyse the utilization of $1\mu\text{mol}$ of substrate per min at 25°C , and one unit of superoxide dismutase activity was as defined by McCord & Fridovich (1969).

Reagents. These were of analytical grade.

Methods

Concentrations of leghaemoglobin. These are expressed as haem concentrations determined by the pyridine haemochrome method (Paul *et al.*, 1953). When degradation of leghaemoglobin was followed, the concentration of residual leghaemoglobin was estimated from the absorption spectra by using the absorption coefficients of the appropriate ligand derivatives (Lehtovaara, 1977).

Absorption spectra. These were recorded with a Cary 15 spectrophotometer in 1 cm quartz microcuvettes (sample volume $200\text{--}250\mu\text{l}$), equipped with beam masks. The cuvette compartment temperature was controlled by thermostat at 20°C or as stated in the text.

Reaction velocity of formation of the green product from leghaemoglobin. This was followed by continuous recording of A_{697} , the 0–0.1 slidewire being used for scale expansion. Other conditions were as described above.

Glyoxylic acid. Identification was based on descending paper chromatography in butanol/pyridine/water (6:4:3, by vol.). Bromophenol Blue (0.05% in water) was used for detection.

Quantitative determinations of glyoxylate were made from reaction mixtures containing $7.9\mu\text{M}$ leghaemoglobin in $500\mu\text{l}$ of 0.2M-glycine/NaOH buffer, pH 9.8. The samples were usually incubated for 90 min in the dark at room temperature (23°C). The reaction was terminated by precipitation of the protein with $200\mu\text{l}$ of 30% (w/v) trichloroacetic acid, after which $600\mu\text{l}$ of the deproteinized supernatant, containing 0.5–8 μg of glyoxylic acid was taken for glyoxylic acid determination (Lui & Roels, 1970). The absorbance was measured against water, and the results were corrected with respect to an identical sample from which the protein had been precipitated immediately.

Digestions with enzymes. The carboxypeptidase A suspension treated with di-isopropyl phosphorofluoridate was washed three times with water and dissolved in 2M- NH_4HCO_3 . Leghaemoglobin was incubated with carboxypeptidase at room temperature (23°C) in borate, Tris or carbonate buffers of pH varying from 8.0 to 9.8. Liberation of amino acids was followed by subjecting portions to paper electrophoresis at pH 1.9 (formic acid/acetic acid/water;

1:4:45, by vol.), at 80 V/cm, and staining the paper with ninhydrin. The digested sample was transferred to glycine buffer by gel filtration. However, digestion in 0.2M-glycine/NaOH buffer, pH 9.8, was the method of choice because it was found to cause the least denaturing of leghaemoglobin.

Superoxide dismutase and catalase were dissolved in 0.2M-glycine buffer, pH 9.8, as concentrated stock solutions immediately before the experiment. Digestions were carried out at room temperature (23°C).

Results

Degradation by alkaline amino acid solutions

Kidney-bean leghaemoglobin is unstable in alkaline amino acid solutions, as shown in Fig. 1. The spectral changes observed included increased absorption in the wavelength ranges 585–750 nm and 473–528 nm, with concomitant decrease in the ranges 300–473 nm and 528–585 nm. Prolonged incubation in glycinate gradually caused denaturation of the protein, and the absorption spectrum lost the isosbestic points observed at the beginning of the reaction. The changes in Fig. 1 are reminiscent of those brought about by the coupled oxidation of haemoproteins. However, coupled oxidation of kidney-bean leghaemoglobin with O_2 and ascorbate (0.1 M-phosphate

Fig. 1. Formation of a green product from kidney-bean leghaemoglobin in glycinate

Leghaemoglobin ($54\mu\text{M}$) was in 0.1 M-glycine/NaOH buffer, pH 9.8, and the incubation temperature was 20°C . The spectra were recorded at the following time intervals from addition of leghaemoglobin to the cuvette: 20 s (that showing the lowest A_{700} value), 4 min, 8 min, 12 min, 16 min, 20 min, 25 min, 30 min, 40 min, 50 min, 75 min, 90 min and 7 h 45 min.

buffer, pH 7.5, containing 2 mg of ascorbic acid/ml) yielded a product with an absorption maximum at 675 nm, whereas incubation in glycinate yielded a different product with a maximum at 697 nm. Dialysis against phosphate buffer, pH 6.0, shifted the 697 nm peak only to 695 nm.

The absorption spectrum of kidney-bean leghaemoglobin remained stable both in Tris and in borate buffer, pH 9.8, for at least 24 h at room temperature, but it was unstable also in amino acids other than glycine. If the relative velocity of the absorption increase at 697 nm in 0.1 M-glycine buffer, pH 9.8, is denoted by 100, the velocities in 0.1 M-serine, 0.1 M-alanine and 0.1 M-valine, all at pH 9.8, were 25, 22 and 14 respectively. Soya-bean leghaemoglobin, human haemoglobin and sperm-whale myoglobin were stable in all these amino acid buffers.

Visible light was found to enhance the rate of formation of the green product. Incubation in day-light instead of in the dark increased the reaction rate to 1.4-fold at room temperature (23°C). A rise in temperature from 23 to 37°C increased the reaction rate to about 2-fold, which obeys the general rule that a reaction approximately doubles for 10°C rise in temperature. However, at an incubation temperature of 4°C, the leghaemoglobin was reduced by glycinate, but the reaction series stopped at this stage. The absorption spectrum was, after 4 days at 4°C, that of pure oxy-leghaemoglobin, and only traces of the green product were present. The activation energy for the steps from oxy-leghaemoglobin to the green product seems to be too high for the reaction to proceed at 4°C.

Nature of the end product

The haem of leghaemoglobin had been transformed, according to the spectral properties, to a mixture of compounds of phlorin and bilatriene type. Because the reaction did not go to completion, the green end product obtained in this work always contained undegraded leghaemoglobin, and removal of this was not attempted. When the apoprotein was detached by acidification with HCl to pH 3, the absorption spectrum with maxima at 642 and 370 nm indicated that biliverdin had been formed by this treatment, even though the yield was very small.

The reaction product consists of the apoprotein attached to a modified haem, which still contains the iron atom. Aromatic residues of the protein seemed not to have been damaged, according to the absorption spectra in the region 250–300 nm. Low-spin ligands were able to shift the characteristic absorption peak of the green product, i.e. in the presence of azide it was shifted to 702 nm and in the presence of cyanide to 691 nm, but the high-spin ligand fluoride had no effect. If carbon monoxide

was added to the green product in glycinate or to the green product dialysed against water, the peak at 697 nm or at 695 nm almost disappeared and a new equally intense peak emerged at about 637 nm.

Effect of the reactant concentrations

Fig. 2 illustrates how the initial velocity of the formation of the green product from leghaemoglobin was followed. This initial velocity was found to be linearly dependent on leghaemoglobin concentration if other conditions were kept constant. The initial velocity decreased if the leghaemoglobin sample was slightly denatured, for example by storage at 4–10°C for some days. A lag phase preceding the linear absorption increase is not revealed by Fig. 1, but it can be observed from Fig. 2. This short lag phase indicates that some other reaction(s) must probably occur before formation of the green product of bile pigment type.

When the glycine concentration was raised, the initial velocity of the formation of the green product was also increased (Fig. 3), but the relationship was not as strictly linear as that of leghaemoglobin concentration and the initial velocity. Raising the glycine concentration over 0.4 M caused decrease of the initial velocity.

The pH-dependence of the reaction is presented in Fig. 4. The optimum pH was about 10.2, and up to

Fig. 2. Measurement of the initial velocity of leghaemoglobin breakdown

Leghaemoglobin (38 μ M) was incubated in a micro-cuvette (light path 1 cm, sample volume 210 μ l) in 0.2 M-glycine/NaOH buffer, pH 9.8, and the absorbance was recorded continuously by using the absorbance full scale 0–0.1. The initial velocity $\Delta A_{697}/\Delta t$ was obtained from the slope of the linear part of the curve after the short lag period, the absolute value of the initial velocity being 1.63×10^{-3} A units/min.

Fig. 3. Effect of glycine concentration on the initial velocity of the formation of the green product

The pH of glycine buffers was 9.8, and leghaemoglobin concentration was $33 \mu\text{M}$. The reaction was followed as in Fig. 2, and the absolute value for the maximal velocity $\Delta A_{697}/\Delta t$ was 1.76×10^{-3} A units/min.

Fig. 4. pH-dependence of the initial reaction velocity of the formation of the green product

The ionic strength of the glycine buffers was 0.1, adjusted with NaCl, and the concentration of leghaemoglobin was $14.3 \mu\text{M}$. The reaction was followed as in Fig. 2, and the absolute value for the minimum velocity $\Delta A_{697}/\Delta t$ was 0.794×10^{-3} A units/min. $\circ$ — $\circ$, Reaction velocity; ---, concentration of unprotonated glycine.

pH 9.7 the initial reaction velocity was directly proportional to the concentration of unprotonated glycine. The decrease in the reaction velocity at high pH values was obviously due to denaturation of the protein. At pH 9.7–10 the enhancement of the reaction velocity was greater than the corresponding increase in the glycinate concentration. This phenomenon indicates ionization(s) in the protein favouring the catabolic reaction, possibly ionization of a tyrosine or a lysine residue.

Reduction of leghaemoglobin

Leghaemoglobin was reduced by glycine before degradation of the haem group. The reduction began immediately, and the O_2 complex was formed. However, the spectral change of ferric hydroxide-leghaemoglobin (maxima at 413, 539 and 572 nm with $10^{-3} \times \epsilon$ values of 110, 12.2 and 11.2 respectively) to the O_2 complex (maxima at 409, 539 and 574 nm; $10^{-3} \times \epsilon$ values of 134, 14.4 and 14.7 respectively) (Lehtovaara, 1977) was difficult to observe, mainly because of the autoxidation of oxy-leghaemoglobin back to the ferric hydroxide form. The reduction was therefore demonstrated as the formation of the

spectrum of the carbon monoxide complex of ferrous leghaemoglobin (absorption maxima at 417, 538 and 563 nm) in glycine buffer saturated with carbon monoxide. Further evidence of the reduction was that addition of pyridine yielded the spectrum of the monopyridine complex of ferrous leghaemoglobin and that, as mentioned above, pure oxy-leghaemoglobin was formed when the incubation was carried out at 4°C .

The increase of A_{697} possibly results from a further reduction of the O_2 complex to some kind of activated oxygen, which then attacks the haem. When the glycine buffer had been bubbled with carbon monoxide before addition of leghaemoglobin, no increase in A_{697} took place. The complexes of ferric leghaemoglobin with azide, cyanide or imidazole were also completely stable in glycinate. These ligands bind strongly to the ferric haem iron even at alkaline pH and thus prevent the formation of the ferrous O_2 complex.

Formation of glyoxylate from glycine

It was demonstrated by paper chromatography that glyoxylate was formed in the reaction of kidney-

bean leghaemoglobin with alkaline glycine buffer. Formation of glyoxylate was followed quantitatively by the phenylhydrazine method. Autoxidation of glycinate to glyoxylate at pH 9.8 was negligible, and protohaem, sperm-whale myoglobin and soya-bean leghaemoglobin were all unreactive in glycine. The formation of glyoxylate by kidney-bean leghaemoglobin ($7.9 \mu\text{M}$ in 0.2M -glycine buffer, pH 9.8) was linear for 1.5–2 h, whereafter the rate of glyoxylate formation gradually decreased. Raising the temperature from 23 to 37°C increased the reaction rate about 2-fold.

The quantitative relationship between glyoxylate formation and degradation of leghaemoglobin indicated that leghaemoglobin was acting as an enzyme. About 10 mol of glyoxylic acid was formed in 1 h at 23°C for every 1 mol of leghaemoglobin present, and because only a minor fraction of the haem was degraded in such a short time, it may be roughly estimated that glycine was reduced to glyoxylic acid in about 100-fold excess compared with the degradation of the haem in leghaemoglobin.

Paper chromatography used for identification of glyoxylate also revealed the presence of remarkable amounts of formate after the reaction between leghaemoglobin and glycine. Unfortunately, the stoichiometry of the different reaction steps cannot be followed, because hydrogen peroxide is formed in the reactions and it promotes a number of rapid side reactions, one of which is oxidation of glyoxylate to formate. Another side reaction is oxidation of glycine to glyoxylic acid in the absence of leghaemoglobin. When 500 nmol of hydrogen peroxide was added to 0.2M -glycine buffer, pH 9.8, 30 nmol of glyoxylic acid was produced in the net reaction. It thus appears that the hydrogen peroxide that is formed during the reaction between leghaemoglobin and glycine reacts with ferrous leghaemoglobin to oxidize the iron, with leghaemoglobin to yield the green product, with glycine to form glyoxylate, and with glyoxylate to yield formate. However, glycine was oxidized in such a great excess, compared with the amount of leghaemoglobin degraded, that the formation of the green product may be regarded as accidental.

Carbon monoxide and azide prevented the formation of the green product, but had little or no inhibitory effect on glycine oxidation (Table 1), indicating that leghaemoglobin can accept electrons also when either of these ligands is bound to the haem iron.

Formation of hydrogen peroxide

The effect of catalase on the degradation of the haem of leghaemoglobin and on the oxidation of glycine to glyoxylate (Fig. 5) shows that hydrogen

Fig. 5. Effect of catalase on the oxidation of glycine to glyoxylate (○), and the effect of catalase (●) and superoxide dismutase (▲) on haem degradation

Oxidation of glycine was followed by the phenylhydrazine method in a solution containing $7.9 \mu\text{M}$ -leghaemoglobin in 0.2M -glycine/NaOH buffer, pH 9.8, and the degradation of haem as in Fig. 2 in a $38 \mu\text{M}$ -leghaemoglobin solution in the same buffer. The enzyme concentrations are expressed as % (w/w) of the amount of leghaemoglobin.

peroxide is involved in both reactions. Neither of the reactions was, however, completely inhibited by catalase, even at high enzyme concentrations.

When the glycine buffer contained carbon monoxide, i.e. the formation of the green product was prevented, glycine was oxidized at a rate that was only 10–15% smaller than without carbon monoxide. This difference was possibly due to decreased partial pressure of oxygen in the solution. Inhibition of glyoxylate formation by catalase was unaffected by the presence of carbon monoxide. These results indicate that hydrogen peroxide is produced also when the haem iron of leghaemoglobin is blocked with carbon monoxide.

Consistently with the inhibition by catalase, formation of the green end product from leghaemoglobin was enhanced by addition of hydrogen peroxide (Fig. 6), and the end product was spectrally identical with that presented in Fig. 1. Recording of the absorbance at 697 nm as a function of time showed that the added hydrogen peroxide was consumed in a few minutes.

Effect of superoxide dismutase

Oxygen may be reduced either by one-electron transfer to the superoxide radical, $\text{O}_2^{\cdot-}$, or directly by two-electron transfer to hydrogen peroxide. If $\text{O}_2^{\cdot-}$ is generated, this will spontaneously dismutate to hydrogen peroxide and oxygen, and this reaction may be accelerated by adding superoxide dismutase to the reaction system.

In the presence of superoxide dismutase the degradation of the haem group was slightly inhibited (Fig. 5). The initial reaction velocity ($\Delta A_{697}/\Delta t$) was about 20% inhibited by superoxide dismutase, but more inhibition was observed from the absorption spectra after a long incubation time. When $72\mu\text{M}$ -leghaemoglobin was incubated in glycine buffer in the absence of superoxide dismutase for 18 h at 23°C in the dark, about 22% of the haem was degraded, whereas only about 12% was degraded in

the same time in the presence of superoxide dismutase (8%, w/w, of the amount of leghaemoglobin). Another difference was that the undegraded part of the leghaemoglobin was mainly in the oxy form when superoxide dismutase was present, as was the case in the presence of catalase. In the same experiment the final amount of glyoxylate was increased to about 1.3-fold by superoxide dismutase.

Effect of carboxypeptidase A

The fact that both carbon monoxide and azide complexes of leghaemoglobin may accept electrons from glycine raises the question of the subsequent fate of the electrons. Furthermore, when these ligands are absent, the formation of the green product may be regarded as accidental, and most of the electrons from glycine seem to be transferred elsewhere than to the haem-bound O_2 . One possibility is that they are mediated to the exterior of the molecule by interacting aromatic residues. The penultimate tyrosine residue, Tyr-144, is present in kidney-bean leghaemoglobin, but not in soya-bean leghaemoglobin, and because this is one of the few remarkable differences between these two proteins, the effect of removal of this tyrosine residue was studied.

It was qualitatively proved by paper electrophoresis that carboxypeptidase A removes, as expected, alanine and tyrosine from kidney-bean leghaemoglobin, although at high enzyme concentrations some lysine and possibly also isoleucine was liberated. However, the digestion was restricted to only a few C-terminal amino acids. The difficulty was to obtain maximal reactivity of the enzyme with minimal denaturation of leghaemoglobin, which are conflicting demands. Direct digestion in glycine buffer was chosen, because it minimized the denaturation.

Carboxypeptidase A was found to activate the formation of the green product from kidney-bean leghaemoglobin (Table 1), whereas soya-bean leg-

Fig. 6. Enhancement of the initial velocity ($\Delta A_{697}/\Delta t$) by added hydrogen peroxide

The leghaemoglobin concentration was $14.3\mu\text{M}$ in 0.2M -glycine/NaOH buffer, pH 9.8. The reaction was followed as in Fig. 2, but the linear part of the curve was short (about 3 min) and no lag period was observed. The added hydrogen peroxide was thus used immediately.

Table 1. Effects of carboxypeptidase A, azide and carbon monoxide on the reaction between kidney-bean leghaemoglobin and glycine

Initial reaction velocity of haem breakdown in 0.2M -glycine/NaOH buffer, pH 9.8, was measured as $\Delta A_{697}/\Delta t$ at 23°C , and formation of glyoxylate in the same buffer at 23°C was followed by the phenylhydrazine method. The carboxypeptidase A (CPA) concentrations are expressed as % (w/w) of the amount of leghaemoglobin.

Haem breakdown (relative initial velocity)		Glyoxylate formation in 90 min (relative value)	
Leghaemoglobin ($37\mu\text{M}$)		Leghaemoglobin ($7.9\mu\text{M}$)	
Without CPA	100	Without CPA	100
With CPA (5.6%)	153	With CPA (20%)	109
With CPA (20%)	264	With CPA (5%) + NaN_3	80
		With CPA (20%) + NaN_3	67
Leghaemoglobin + CO	0	Leghaemoglobin + CO	85-90
Leghaemoglobin + NaN_3	0	Leghaemoglobin + NaN_3	100

haemoglobin yielded no green product in glycine buffer even in the presence of this enzyme. An overnight incubation of kidney-bean leghaemoglobin with and without carboxypeptidase showed that the enzyme had increased the amount of green product to about 2-fold.

Carboxypeptidase did not inhibit the concomitant formation of glyoxylate. However, when the iron of leghaemoglobin was blocked with azide, which is not inhibitory to carboxypeptidase (Pétra, 1970), this enzyme partly inhibited the formation of glyoxylate (Table 1). These results indicate that, if the haem group is not blocked, leghaemoglobin accepts electrons from glycine regardless of whether the C-terminal end of the molecule is present. Removal of C-terminal amino acids enhances reactions that damage the haem. When the haem group is blocked, leghaemoglobin also accepts electrons from glycine, provided that the C-terminal end is intact. If the haem group is blocked and the C-terminal end is removed from several of the leghaemoglobin molecules, inhibition of glyoxylate formation is finally observed.

Discussion

Several factors may contribute to the unique reactivity of kidney-bean leghaemoglobin with glycinate. It has been shown that its haem group is much more accessible to bulky ligands than are the haem groups of other related proteins (Lehtovaara, 1977), and the present results on reaction rates with different amino acids also suggest that steric restrictions might play a role in the reactions described here. The pH-dependence of the formation of the green product from leghaemoglobin suggests that the reaction might be enhanced by ionization of a tyrosine or a lysine residue. In addition to the distribution of the aromatic amino acids, the residue E-10, which is serine (Ser-64) in kidney-bean leghaemoglobin but lysine (Lys-64) in soya-bean leghaemoglobin, is one of the main differences between these two proteins (Lehtovaara & Ellfolk, 1975; Ellfolk & Sievers, 1971, 1974). The residue E-10 is presumably situated beside the haem crevice towards the solvent.

Oxidation and reduction of cytochromes has been studied to elucidate the electron-transport mechanism and to correlate it with the known primary structures. An electron-transfer role for the Tyr-74 residue of cytochrome *c* has been proposed (Takano *et al.*, 1973), but it has lately been abandoned (Dickerson & Timkovich, 1975, p. 483), and at present this redox mechanism remains unresolved. Electron transfer of cytochrome *c* is thought to proceed in two ways: via the protein and to the haem directly (Shafferman & Stein, 1977). Studies on cytochrome *c*₂ indicate that electron transfer seems to take place at the exposed haem edge (Wood *et al.*, 1977), from which it follows

that the amino acid sequence near this region should affect the rate of electron transport. Similarly, the possibility remains that the Ser/Lys difference in the haem environment may affect the electron-accepting capacity of the two leghaemoglobins.

The alkaline conditions of the reactions studied here may be expected to be mildly denaturing to leghaemoglobin. However, no severe denaturation can have taken place, because this would have prevented the whole reaction at the first, reductive step. It was also observed that a leghaemoglobin preparation, which according to circular-dichroic spectra was slightly denatured, formed the green product in glycinate much more slowly than did the native protein. The ionization of the penultimate tyrosine in alkaline haemoglobin solutions may lead to uncoiling from the C-terminus (Perutz, 1974). If the native state of leghaemoglobin is similarly stabilized by the C-terminal tyrosine, Tyr-144, the accessibility of the haem group to solvent might be increased by ionization or removal of this tyrosine residue. Extensive digestion of soya-bean leghaemoglobin with carboxypeptidase did not bring about any reaction between its haem and glycinate.

The degradation of kidney-bean leghaemoglobin in glycinate implies that the iron atom must be able to react with O₂. The results indicate that an 'activated oxygen' is formed in the reduction of oxy-leghaemoglobin, and hydrogen peroxide was shown to be produced. Superoxide radical may also participate at some level, and the ability of superoxide dismutase to preserve oxy-leghaemoglobin is presumably due to inhibition of the reaction (Sutton *et al.*, 1976):

The fact that neither catalase nor superoxide dismutase was capable of totally preventing the formation of the green product may reflect the inability of these enzymes to compete efficiently with leghaemoglobin for the active form of oxygen. It is also possible that the damaging reactant is the hydroxyl radical, which is generated in the Haber-Weiss reaction between superoxide radical and hydrogen peroxide (Haber & Weiss, 1934; Fridovich, 1976):

The hydroxyl radical is an extremely potent oxidant, which may initiate haem breakdown by a hydroxylation reaction.

According to the reaction mechanism outlined for haem catabolism by O'Carra (1975) and Brown (1976), the activated oxygen might be a reduction product of the haem-O₂ complex, possibly a peroxide or a superoxide bound to the iron. The present results appear substantially to support their views. Further-

more, the formation of the green product from leghaemoglobin is such a slow reaction, compared with the rapid oxidation of glycinate, that elimination of the injurious electrons by some protective pathway may be the normal event and attack of the haem group may be accidental.

It is essential that the haem group of reversible O₂ carriers is well protected not only against oxidation, but also against reduction. Activation of the catabolic reaction of kidney-bean leghaemoglobin by carboxypeptidase might suggest a role for Tyr-144 as a part of a protective, alternative route for electrons. The final fate of the electrons remains unknown, but they might react with some solvent component, e.g. with O₂ to form hydrogen peroxide. The electron-accepting capacity of leghaemoglobin was not impaired before the haem was blocked with a ligand preventing the formation of the O₂ complex, and C-terminal amino acids were simultaneously removed by carboxypeptidase. The present results might be explained by a hypothesis that injurious electrons are eliminated from the haem site to the exterior of the molecule via the protein, Tyr-144 being an essential part of the protective mechanism.

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