OXIDATION OF CYTOCHROME C PEROXIDASE WITH HYDROGEN PEROXIDE: IDENTIFICATION OF THE 'ENDOGENOUS DONOR'

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SUMMARY

The decay of the 'ES compound' of cytochrome c peroxidase to the native ferric enzyme involves destruction of amino-acid residues. At pH 4 and pH 8, tyrosine and tryptophan are the residues principally affected. The loss of 3-4 tyrosines is accompanied by loss of enzyme activity.

INTRODUCTION

Cytochrome c peroxidase (EC 1.11.1.5, 'ccp') has an obligatory order mechanism (1), of which the first stage is oxidation of the protein by hydrogen peroxide to form the so-called 'ES compound'. In common with horse radish peroxidase and related enzymes, ES will decay back to the native, ferric state in the absence of added reducing agent, and this is attributed to the presence of an endogenous donor. After several cycles of formation and decay, the enzyme loses activity. The endogenous donor in horse radish peroxidase has attracted a good deal of attention, but because of the high molecular weight of the enzyme, its heterogeneity and the presence of polysaccharide, the reducing agent has not been identified. Ccp is free of these technical disadvantages. We show here that in this process amino-acid residues of ccp are modified and we identify the residues principally affected.

MATERIALS AND METHODS

Ccp was prepared as described previously (2). Solutions of hydrogen peroxide were made by dilution of Merck 'Superoxol' (30% hydrogen peroxide) with water. Paratoluenesulphonic acid, purified by the method of Liu and Chang (3),

was kindly provided by Mr. Koji Takio. Tryptamine was prepared from Eastman tryptamine hydrochloride, and was recrystallized to constant melting point from benzene/n-hexane.

U-v and visible spectral measurements were made on a Cary model 14, a Perkin-Elmer model 323, or on a Perkin-Elmer model 124, fitted with a scale expander and pen recorder. pH measurements were carried out with a Sargent-Welch model NX digital pH meter. Amino-acid analysis was carried out on a Jeolco 5AH analyzer, using the Durrum 'Picobuffer' single column analysis system, or, for the later tryptophan analyses, an 0.8 x 12 cm column of Jeolco type AR-15 resin developed at pH 5.28.

Amino-acid analysis. Hydrolysis in 6N HCL was carried out in vacuo at 108° for 24 hours in re-usable glass tubes fitted with O-ring seals and taps (Kontes K-896850). Performic acid oxidation was carried out by the method described by Hirs (4). Alkaline hydrolysis was carried out by the modification already described (5) of the technique of Oehlshlegel et al (6). Attempts were made to use the unmodified technique of these authors, but it was found that even samples diluted as recommended contained enough salt to interfere with the short column analysis and prevent the resolution of tryptophan and lysine.

Tryptophan analysis was also carried out by the paratoluenesulphonic acid method of Liu and Chang (3). Tryptamine (2.5-3.5 mg) and 3N p-toluenesulphonic acid (1 ml) were added to each sample of protein (about 1.7 mg). The mixtures were frozen, the tubes evacuated and the samples allowed to melt and degas gently. The tubes were sealed at 5-10μ Hg, and incubated at 108° for 20-24 hours.

Incubation of ccp with hydrogen peroxide. All incubations were carried out by addition of suitable small volumes of 1 mM hydrogen peroxide to 10 μM solutions of ccp in 0.1M phosphate, pH 7 or 0.1M or 20mM acetate buffer pH 4. The reaction mixtures were allowed to stand at room temperature for 24 hours, dialysed against distilled water (for the samples containing the higher salt concentration), lyophilized and analyzed.

AMINO-ACID	MOLES H_2O_2/MOLES PROTEIN 0	1	10
Lys	22.5	22.2	21.5
His	5.4	5.7	5.5
Arg	10.0	10.1	9.9
Asp	41	41	41
Thr	14.2	14.5	14.2
Ser	13.6	14.0	14.3
Glu	27.7	27.9	27.5
Pro	15.8	15.5	15.9
Gly	23.9	23.9	24.5
Ala	18.3	18.8	18.9
Val	13.0	12.8	11.9
Met	3.6	4.7	4.6
Ile	8.7	8.9	8.9
Leu	23.4	23.4	22.6
Tyr	11.9	11.4	8.5
Phe	16.0	15.5	14.5

Table I. Amino-acid composition (residues/mole) of acid hydrolysates of samples of ccp incubated with hydrogen peroxide under conditions given in the text. The numbers of residues are normalized to Asp=41 (5) and each figure is the average of the results obtained in three identical experiments.

RESULTS

Amino-acid residues destroyed by incubation of ccp with hydrogen peroxide at pH 7. Samples of ccp were incubated with a 0, 1, and 10-fold molar excess of hydrogen peroxide. Table 1 shows the composition of the amino-acid mixtures obtained on acid hydrolysis of these samples. Each figure is the mean obtained from three identical experiments. Only tyrosine shows a substantial reaction, 3.5 residues (=30%) being destroyed after reaction at the higher concentrations of hydrogen peroxide. Up to about one residue each of lysine, valine, and phenylalanine may be destroyed at this pH, but it is hard to be sure that these changes are significant.

The single cysteine residue of ccp (5) is apparently stable under this treatment. No cysteine or cysteic acid was detected in the acid hydrolysates, and the number of cysteic acid residues recovered after performic acid oxidation of samples incubated with 0, 1 and 10-fold molar excess of hydrogen peroxide were as follows (mean of values relative to Asp, Glu and Leu): 1.05, 1.00, 1.05.

In the analysis system used here, methionine sulphoxide and methionine sulphone are not clearly resolved, but are eluted about 2 minutes before aspartic acid and 3 minutes before threonine respectively. Methionine sulphone was not detected in the acid hydrolysates, and methionine sulphoxide was not detected in the alkaline hydrolysates used for tryptophan analysis; nor was there any apparent increase in the peak widths of aspartic acid and threonine.

Tryptophan was determined after alkaline hydrolysis of similar samples. The number of residues of tryptophan (mean of values relative to Asp, Glu , and Phe) after incubation with 0, 1 and 10-fold excess of hydrogen peroxide were

	MOLES H_2O_2/MOLES PROTEIN		
AMINO-ACID	0	1	10
Lys	21.3	21.3	21.4
His	5.4	5.3	5.3
Arg	9.1	9.1	9.1
Asp	41	41	41
Thr	14.3	14.2	14.1
Ser	13.2	13.1	13.1
Glu	26.1	25.7	25.8
Pro	16.6	15.6	15.6
Gly	22.2	22.6	22.7
Ala	16.8	16.9	15.6
Val	12.1	11.7	11.7
Met	4.0	4.2	4.3
Ile	7.8	8.0	8.2
Leu	22.4	22.1	22.0
Tyr	12.4	11.5	8.4
Phe	16.3	16.3	16.4

Table II. Amino-acid composition (residues/mole) of acid hydrolysates of samples of ccp incubated with hydrogen peroxide under conditions given in the text. The numbers of residues were normalized to Asp=41, and each figure is the mean of the results of two identical experiments.

6.2, 6.0 and 5.6 respectively.

Amino-acids destroyed by incubation of ccp with hydrogen peroxide at pH 4. Similar experiments were performed at this pH. Table 2 shows the amino-acid compositions of the acid hydrolysates of the peroxide-modified protein.

The results are similar to those obtained at pH 7, although more clear-cut. Once again there is extensive destruction only of tyrosine. Like the small changes observed at pH 7, the apparent losses of up to one residue each of the proline and alanine may not be significant. Once again, no cysteine or methionine oxidation products were observed in either acid or alkaline hydrolysates, and it was concluded that these residues are unaffected.

Tryptophan was analyzed in samples subjected to the p-toluenesulphonic acid hydrolysis method of Liu and Chang (3). The yield of tryptophan from unmodified protein was similar to that obtained by these authors with other proteins after 24 hours hydrolysis, and similar also to that obtained by alkaline hydrolysis of ccp (about 90% in each case). The number of residues (mean of values compared to Lys and Arg) after incubation at pH 4 with 0, 1 and 10-fold molar excess of hydrogen peroxide were as follows (each figure is the mean of two identical experiments): 5.9, 5.3, 3.9.

Correlation of loss of activity and destruction of tyrosine residues at pH 4. 10 μM solutions of ccp were incubated with 0, 1, 2, 5, 7 and 10 moles of H_2O_2/mole ccp at pH 4 under the usual conditions. 2 ml of each sample were taken and the spectrum in the region of the Soret band observed before and after addition of a small excess of hydrogen peroxide (determined by prior titration with native ccp). The enzymic activity was expressed as the ratio of the change in absorbance at 420 nm to the original absorbance at 408 nm. Further aliquots of each reaction mixture were lyophilized and analyzed in the usual way. Figure 1 shows the relation between the sumber of residues of tyrosine recovered (each figure is the mean of values compared to His, Val, Phe and Arg) and the activity of the modified protein. The result for the unmodified protein is the mean of three similar experiments.

DISCUSSION

The decay reaction of ES has been carefully studied by J.E. Erman (7), who has shown that similar reactions occur when ccp is taken through several cycles of formation and decay whether the same total excess of H_2O_2 is added

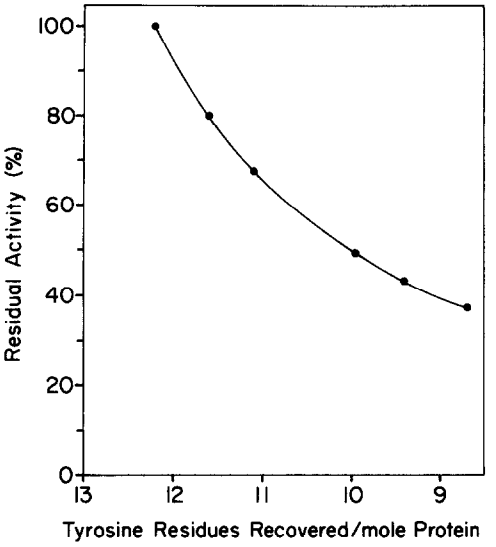

Fig. 1. Residual activity of ccp (measured as capacity to react with hydrogen peroxide to form ES compound) as a function of the number of tyrosine residues recovered after acid hydrolysis of the modified protein.

at once or in equimolar aliquots. He also showed that reaction was complete within 24 hours, and these two facts dictated the choice of reaction conditions employed here. The oxidation of proteins with hydrogen peroxide has been briefly reviewed by Neumann (8). The reaction generally requires protein concentrations a hundred times greater, and peroxide concentrations a thousand times greater than those employed here. Furthermore, the residues destroyed most rapidly are methionine and cysteine, while tyrosine and tryptophan are oxidised slowly or not at all.

In the case of ccp, tyrosine and tryptophan are the only residues certainly modified at pH 4 and pH 7, and no reaction of cysteine or methionine could be detected. The destruction of tyrosine is rapid and extensive. About 30% of the total tyrosine was destroyed by incubation with 10 moles H_2O_2/mole ccp. These facts suggest strongly that the protein modification is a specific process mediated exclusively by the ES compound of the enzyme. We can therefore predict that a high proportion of the aromatic amino-acids (at least 2 of the tryptophans

and 4 of the tyrosines) in ccp have immediate access to oxidising equivalents associated with the heme iron, and may be spacially immediately adjacent to the heme ring.

There are two differences between the products of reaction at pH 4 and pH 7. The two or three amino-acids which show changes of about one residue after oxidation with 10 moles hydrogen peroxide/mole ccp are different at these two pH's, and this implies that these apparent changes are not significant. Secondly, the destruction of tryptophan is far more extensive at the lower pH (about 1.5 instead of 0.5 residues out of 6 in all). This change in susceptibility of tryptophan with change in pH is not easy to reconcile with the enzyme mechanism having an absolute requirement for this amino-acid.

It is tempting to speculate that tyrosine is involved in the activity of the enzyme, and in particular that the free-radical-like epr signal in ES is due to a tyrosyl radical, but the relation between the destruction of tyrosine and the loss of activity cannot be interpreted as showing a requirement for one or a small number of essential tyrosines. The simplest explanation may be that continued destruction of tyrosine and tryptophan residues in the vicinity of the heme causes progressive structural changes that lead eventually to loss of activity.

The susceptibility of ccp to oxidation in the neighborhood of the heme has been studied previosly by photo-oxidation of apo-ccp in the presence of a sensitising dye which binds to the heme binding site (5). In that case, histidine and tryptophan were destroyed. The fact that histidine was not destroyed in the experiments described here suggests that it may be shielded by the heme (as it would be if it were a heme ligand). It was suggested earlier that the oxidised tryptophan might exercise a protective function for the enzyme by absorbing oxidising equivalents without allowing a total loss of activity. Our present experiments certainly show that a cluster of tyrosines and tryptophans around the heme, whether or not they are directly involved in the activity, can absorb oxidising equivalents to a considerable extent before the activity is lost.

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