Proteolytic and Peroxidatic Reactions of Commercial Horseradish Peroxidase with Myelin Basic Protein

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Degradation of myelin basic protein during incubations with high concentrations of horseradish peroxidase has been demonstrated [Johnson & Cammer (1977) *J. Histochem. Cytochem.* **25**, 329-336]. Possible mechanisms for the interaction of the basic protein with peroxidase were investigated in the present study. Because the peroxidase samples previously observed to degrade basic protein were mixtures of isoenzymes, commercial preparations of the separated isoenzymes were tested, and all three degraded basic protein, but to various extents. Three other basic proteins, P₂ protein from peripheral nerve myelin, lysozyme and cytochrome *c*, were not degraded by horseradish peroxidase under the same conditions. Inhibitor studies suggested a minor peroxidatic component in the reaction. Therefore the peroxidatic reaction with basic protein was studied by using low concentrations of peroxidase along with H₂O₂. Horseradish peroxidase plus H₂O₂ caused the destruction of basic protein, a reaction inhibited by cyanide, azide, ferrocyanide, tyrosine, di-iodotyrosine and catalase. Lactoperoxidase plus H₂O₂ and myoglobin plus H₂O₂ were also effective in destroying the myelin basic protein. Low concentrations of horseradish peroxidase plus H₂O₂ were not active against other basic proteins, but did destroy casein and fibrinogen. Although high concentrations of peroxidase alone degraded basic protein to low-molecular-weight products, suggesting the operation of a proteolytic enzyme contaminant in the absence of H₂O₂, incubations with catalytic concentrations of peroxidase in the presence of H₂O₂ converted basic protein into products with high molecular weights. Our data suggest a mechanism for the latter, peroxidatic, reaction where polymers would form by linking the tyrosine side chains in basic-protein molecules. These data show that the myelin basic protein is unusually susceptible to peroxidatic reactions.

Basic protein isolated from central-nervous-system myelin is the antigen for the autoimmune disease experimental allergic encephalomyelitis. This disease has been studied for many years as a model for possible immunological aspects of multiple sclerosis. A number of immunohistochemical studies have been carried out with glutaraldehyde conjugates of basic protein with horseradish peroxidase in an attempt to reveal specific anti-(basic protein) antibodies in plasma cells in lymph nodes and the nervous system of animals with experimental allergic encephalomyelitis and experimental allergic neuritis (Dal Canto *et al.*, 1974, 1975; Gonatas *et al.*, 1974; Johnson & Blum, 1972; Johnson *et al.*, 1971; Whitaker, 1976), and in tissues of patients with multiple sclerosis and Guillain-Barré syndrome (Johnson & Dal Canto, 1976). During studies to check the effectiveness of the conjugation of horseradish peroxidase (EC 1.11.1.7; donor-H₂O₂ oxidoreductase) with basic protein, it was observed that basic protein was

degraded in control mixtures of the protein and peroxidase incubated in the absence of the usual cross-linking agent, glutaraldehyde (Johnson & Cammer, 1977). Although this phenomenon was described only in terms of practical methodological consequences, two alternatives for hypothetical mechanisms were proposed, one involving the possibility that the peroxidase samples were contaminated with proteolytic enzymes, and the other suggesting the possibility of peroxidatic attack on basic protein by peroxidase with an endogenous peroxide presumably bound to its active site. The present investigation was designed to resolve the question of one or more mechanisms for the destruction of native basic protein by commercial preparations of horseradish peroxidase. We conclude that when basic protein is incubated for 1 h or more with the enzyme alone (molar ratio basic protein/peroxidase approx. 1:1), the basic protein is probably attacked by contaminating proteinases in the peroxidase preparations, yielding peptides of low molecular weight. However, when basic protein

is incubated with catalytic amounts of peroxidase (molar ratio basic protein/peroxidase approx. 4:1) plus H_2O_2 for 30 min, a peroxidatic reaction destroys the native basic protein and yields higher-molecular-weight products. A mechanism for the peroxidatic reaction is proposed, involving formation of polymers by linking the tyrosine side chains in basic-protein molecules. Inhibitor studies are presented which support that interpretation.

Materials and Methods

Myelin was prepared from ox, cat, rabbit and rat brains as described by Norton & Poduslo (1971). It was delipidated and basic protein prepared as described previously (Johnson & Cammer, 1977). For disc gel electrophoresis on acid (pH 4.5) gels, 7.5% (w/v) polyacrylamide gels were prepared by the method of Reisfeld *et al.* (1962), except that the gels were 10 cm long and contained 4 M-urea. Immediately before beginning electrophoresis, incubation mixtures were diluted 1:1 with 8 M-urea, and 100 μ l portions were placed on the gels. After electrophoresis (for details of conditions, see the Figure legends) the gels were fixed and stained in 1% Fast Green in methanol/water/acetic acid (9:9:2, by vol.) and were destained in 10% (v/v) acetic acid. The gels were scanned in a Gilford spectrophotometer at 580 nm. Electrophoresis on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulphate was performed as described by Greenfield *et al.* (1971).

Samples of horseradish peroxidase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). The Sigma type VI and Worthington horseradish peroxidase samples used previously (Johnson & Cammer, 1977) and in most of the experiments described here contain mixtures of the acidic and basic isoenzymes, whereas the Sigma types VII, VIII and IX, which became available more recently, are the separated isoenzymes. Cytochrome *c*, lysozyme, histone, polylysine, catalase, myoglobin, fibrinogen, casein and β -globulins were purchased from Sigma, and a sample of the basic P_2 protein from peripheral myelin, prepared by Dr. S. Brostoff (Charleston, SC, U.S.A.) from bovine spinal roots, was a gift from Dr. A. B. Johnson (Bronx, NY, U.S.A.). Lactoperoxidase was purchased from Boehringer Mannheim Corp. (Indianapolis, IN, U.S.A.). H_2O_2 , as a 30% (v/v) solution purchased from Mallinckrodt Chemical Co. (St. Louis, MO, U.S.A.), was diluted 1:2000 with cold water immediately before portions were taken for the reaction with peroxidase and basic protein. The H_2O_2 was standardized by the method of Cotton & Dunford (1973). The reaction mixtures and peroxidase solutions contained phosphate-buffered saline consisting of 10 mM-sodium phosphate, pH 7.2, plus 150 mM-NaCl. Although not used in the experiments reported

here, similar results were obtained when 0.1 M-sodium phosphate buffer, pH 7.2, was substituted for phosphate-buffered saline.

Results

Effect of horseradish peroxidase on basic protein in the absence of H_2O_2

The peroxidase samples previously observed (Johnson & Cammer, 1977) to degrade basic protein to low-molecular-weight products consisted of mixtures of isoenzymes. Therefore when preparations of the separated isoenzymes became available, their ability to degrade basic protein was tested. Types VII and VIII are acidic isoenzymes, and type IX is the basic isoenzyme. In the experiments reported previously (Johnson & Cammer, 1977) a large excess of peroxidase over basic protein was used (peroxidase/basic-protein molar ratio equal to 6:1), but less enzyme was used in the studies reported here, so that differences among the peroxidase preparations could be resolved and the effects of inhibitors determined. Each of these peroxidase preparations was incubated with basic protein for 1 h at peroxidase/basic-protein molar ratios of 0.5:1 and 1.6:1, and the products were examined on acid (pH 4.5) urea gels (Fig. 1*a*). Untreated basic protein gave a darkly staining band on the acid urea gels (Fig. 1*a*, gels 1 and 2), but, after incubation with peroxidase, the native basic-protein bands became less prominent (gels 3–6 and 8). The numbers shown above the gels in Fig. 1*a* for percentage of basic protein degraded were obtained by spectrophotometric scanning of the gels and measurement of the areas under the basic-protein bands, and they indicate that the isoenzyme preparations all degraded the basic protein, but to differing extents, during the 1 h incubation. Bands from the basic peroxidase isoenzyme are visible near the tops of gels 7 and 8, but, apparently, the acid isoenzymes (gels 3–6) do not enter the acid urea gels. Our previous lots of Sigma horseradish peroxidase (all type VI) have shown heavily staining bands on acid urea gels [Johnson & Cammer (1977) and Figs. 1*b*–1*d*], indicating that they contain largely the basic isoenzyme, as is asserted in the Sigma catalogue.

Vulnerability to horseradish peroxidase preparations may be a property of the myelin basic protein that is not found in most other proteins. Two other basic proteins, lysozyme and cytochrome *c*, were resistant to peroxidase effects (Fig. 1*b*), and a basic protein, P_2 , isolated from peripheral nerve myelin, also was not degraded (Fig. 1*c*, gel 1), whereas basic protein from rat brain myelin was degraded completely (Fig. 1*c*, gel 4). This result was not surprising, since the basic proteins of rat brain myelin consist of the common 18000-mol.wt. protein along with a 14000-mol.wt. chain derived from the 18000-mol.wt.

Fig. 1. Effects of concentrated horseradish peroxidase alone on myelin basic protein

Horseradish peroxidase and basic protein or other proteins were incubated in phosphate-buffered saline (total volume 50 μ l) at room temperature for the indicated times, 50 μ l of 8M-urea was added, and the mixtures were electrophoresed on acid urea gels. (a) Effects of horseradish peroxidase isoenzymes on basic protein. Each incubation mixture contained: 30 μ g of cat brain basic protein, plus the following additions: 1 and 2, none; 3, 35 μ g of Sigma type VII peroxidase; 4, 105 μ g of type VII peroxidase; 5, 35 μ g of type VIII peroxidase; 6, 105 μ g of type VIII peroxidase; 7, 35 μ g of type IX peroxidase; 8, 105 μ g of type IX peroxidase. Incubation time was 1 h, and electrophoresis was at 70mA for 90min. The numbers shown above the gels indicate the percentage of basic protein degraded. (b) Effects of horseradish peroxidase on lysozyme and cytochrome *c*. Incubation mixtures contained: 1, 30 μ g of lysozyme; 2 and 3, 30 μ g of lysozyme plus 240 μ g of peroxidase (type VI); 4, 60 μ g of cytochrome *c*; 5 and 6, 60 μ g of cytochrome *c* plus 240 μ g of peroxidase. Incubation time was 3.5h, and electrophoresis was for 70min at 70mA. (c) Effects of horseradish peroxidase on P₂ protein from peripheral-nervous-system myelin and basic protein from rat brain myelin. Incubation mixtures contained: 1, 30 μ g of P₂ protein plus 240 μ g of peroxidase (Sigma type VI), incubated 4h; 2, 30 μ g of P₂ protein incubated 4h; 3, 30 μ g of P₂ protein plus 240 μ g of peroxidase, zero time; 4, 50 μ g of rat brain basic protein plus 240 μ g of peroxidase, incubation 4h; 5, 50 μ g of rat brain basic protein, incubation 4h; 6, 50 μ g of rat brain basic protein plus 240 μ g of peroxidase, zero time. Electrophoresis was at 50mA for 90min. (d) Inhibitor effects on incubation of basic protein with horseradish peroxidase alone: 1 and 2, 30 μ g of basic protein alone; 3-8, 30 μ g of basic protein plus 48 μ g of type VI peroxidase; other additions: 5, 12mM-KCN; 6, 6mM-NaN₃; 7, 7mM-NaF; 8, peroxidase was boiled for 2min. Incubation time was 90min; electrophoresis was for 2h at 70mA.

Fig. 2. Effect of H_2O_2 plus Worthington horseradish peroxidase on myelin basic protein and other proteins

In (a), (b) and (c) the incubation mixtures (total volume 55, 50 and 50 μ l respectively, in phosphate-buffered saline) were incubated for 30 min at room temperature, 50 μ l of 8M-urea was added, and the samples were electrophoresed for 90 min at 50 mA on acid urea gels. (a) Effect of varying the peroxidase concentration. Incubation mixtures contained 1, 30 μ g of bovine brain myelin basic protein; 2, 30 μ g of basic protein plus 24 μ g of horseradish peroxidase; 3, 30 μ g of basic protein plus 6 μ g of peroxidase plus 0.68 mM- H_2O_2 ; 4, 30 μ g of basic protein plus 12 μ g of peroxidase plus 0.68 mM- H_2O_2 ; 5, 30 μ g of basic protein plus 18 μ g of peroxidase, plus 0.68 mM- H_2O_2 ; 6, 30 μ g of basic protein plus 24 μ g of peroxidase plus 0.68 mM- H_2O_2 . (b) Effect of varying the H_2O_2 concentration. All samples contain 30 μ g of cat brain myelin basic protein; 2-6 contain 18 μ g of horseradish peroxidase; 3-6 contain H_2O_2 as follows: 3, 0.14 mM; 4, 0.28 mM; 5, 0.42 mM; 6, 0.56 mM. (c) Effect of horseradish peroxidase plus H_2O_2 on P_2 protein and lysozyme. 1, 30 μ g of lysozyme; 2, 30 μ g of lysozyme plus 0.62 mM- H_2O_2 ; 3, 30 μ g of lysozyme plus 0.62 mM- H_2O_2 plus 300 μ g of peroxidase; 4, 40 μ g of P_2 protein; 5, 40 μ g of P_2 protein plus 0.62 mM- H_2O_2 ; 6, 40 μ g of P_2 protein plus 0.62 mM- H_2O_2 plus 300 μ g of peroxidase. (d) Effect of horseradish peroxidase plus H_2O_2 on fibrinogen, casein and β -globulins and effect of lactoperoxidase plus H_2O_2 on basic protein. The following mixtures (total volume 50 μ l) were incubated at room temperature for 4 h. Subsequently 5 μ l of 10% sodium dodecyl sulphate was added and the mixtures were boiled for 2 min. Sample solvent (Greenfield *et al.*, 1971) (50 μ l) was added along with solid urea, to give a final concentration of 4M-urea, and the samples were incubated for 0.5 h at 45°C. The samples were electrophoresed on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Greenfield *et al.*, 1971) for 18 h at 45 V and were fixed, stained and destained (Greenfield *et al.*, 1971). Reaction mixtures contained: 1, 30 μ g of basic protein; 2, 30 μ g of basic protein plus 18 μ g of horseradish peroxidase plus 0.62 mM- H_2O_2 ; 3, 40 μ g of lactoperoxidase; 4, 30 μ g of basic protein plus 40 μ g of lactoperoxidase; 5, 30 μ g of basic protein plus 40 μ g of lactoperoxidase plus 0.62 mM- H_2O_2 ; 6, 60 μ g of casein; 7, 60 μ g of casein plus 18 μ g of horseradish peroxidase plus 0.62 mM- H_2O_2 ; 8, 30 μ g of fibrinogen; 9, 30 μ g of fibrinogen plus 18 μ g of horseradish peroxidase plus 0.62 mM- H_2O_2 ; 10, 60 μ g of β -globulins; 11, 60 μ g of β -globulins plus 18 μ g of horseradish peroxidase plus 0.62 mM- H_2O_2 ; 12, 30 μ g of basic protein.

protein (Martenson *et al.*, 1972). The acid urea gels resolved more than two bands in rat brain basic protein (gels 5 and 6), indicating differences in charge as well as molecular weight, and all the bands disappeared during incubation with Sigma peroxidase.

Our hypothetical mechanisms (Johnson & Cammer, 1977) for the rapid degradation of basic protein by horseradish peroxidase were that the enzyme samples were contaminated with a proteinase, or alternatively, that the peroxidase had endogenous peroxide bound to its active site. The possibility that the breakdown of basic protein by the Sigma enzyme was peroxidatic was tested by using classic peroxidase inhibitors (Fig. 1*d*). In this experiment a relatively low concentration of the most active peroxidase preparation (Johnson & Cammer, 1977; Sigma lot B) was used to reveal possible inhibitor effects on the reaction rate. Of cyanide, fluoride and azide, only azide retarded basic-protein degradation, and over 90% of the basic protein was destroyed even in the presence of azide. This degree of inhibition was small but reproducible. Boiling the peroxidase for 2 min destroyed 50% of its activity, confirming the enzymic nature of basic-protein degradation. Pre-incubation of the peroxidase with 225 μ g of catalase, which would be expected to destroy endogenous H_2O_2 , yielded only 18% inhibition of basic-protein breakdown. These results suggested that the mechanism of basic-protein breakdown by peroxidase alone (no H_2O_2 added) during incubation for 1 h was enzymic but not predominantly peroxidatic. However, the slight inhibitory effects of catalase and azide stimulated an investigation of a possible small peroxidatic contribution to basic-protein degradation.

Effect of catalytic concentrations of horseradish peroxidase on basic protein in the presence of H_2O_2

Horseradish peroxidase from the Worthington Co. was chosen for use in a study of the peroxidatic reaction because when used alone it is relatively slow in degrading basic protein (Johnson & Cammer, 1977). When 16 μ g of basic protein was incubated overnight with 240 μ g of the Worthington enzyme, paralleling the conditions used for coupling basic protein to peroxidase, the basic protein was degraded, but an incubation time of 0.5 h left much of the basic protein intact (Johnson & Cammer, 1977). We concluded that the Worthington peroxidase contained less of the proteolytic-enzyme contaminant than did the preparations from Sigma.

Disappearance of the native basic-protein band was accelerated when H_2O_2 was added to mixtures of basic protein along with various concentrations of Worthington peroxidase (Fig. 2*a*, gels 3–6). In the presence of 0.68 mM- H_2O_2 , 24 μ g of peroxidase destroyed over 90% of the basic-protein band in 0.5 h (gel 6), whereas 24 μ g of the enzyme destroyed

only 25% of the basic-protein band in 0.5 h when no H_2O_2 was added (gel 2). The concentration of H_2O_2 affected the degree of disappearance of the basic-protein band (Fig. 2*b*) when 18 μ g of peroxidase was used, and less than 0.2 mM- H_2O_2 had a detectable effect. Low concentrations of Sigma types VI–IX peroxidase, instead of the Worthington enzyme, had similar effects on basic protein in 30 min incubations with 0.68 mM- H_2O_2 .

A difference in the products of peroxidase activity toward basic protein was suspected on the basis of the results from the acid urea gels shown in Figs. 1*(a)* and 1*(d)* as compared with Figs. 2*(a)* and 2*(b)*. When Sigma peroxidase preparations were incubated for 1 h with basic protein in the absence of H_2O_2 , breakdown products appeared below basic protein on acid urea gels (Figs. 1*a* and 1*d*) and sodium dodecyl sulphate/polyacrylamide gels (Johnson & Cammer, 1977). In Fig. 1*(d)* the heavily stained peroxidase bands on top of the gels make it difficult to detect possible high-molecular-weight products, but in Fig. 1*(a)* (gels 3–6) there are clearly no products above basic protein on the gels. A very different pattern was found for the peroxidatic reaction in Figs. 2*(a)* and 2*(b)*, where the only low-molecular-weight material occurred as a contaminant in the native basic protein and material accumulated between the basic protein and peroxidase bands (Figs. 2*a* and 2*b*, gels 3 and 4; arrows in Fig. 2*b*) during the reaction. When the basic-protein band had almost disappeared (Figs. 2*a* and 2*b*, gels 5 and 6), the bands between basic protein and peroxidase also faded, and stain accumulated above peroxidase on the gels (Fig. 2*b*, gels 4–6). When the gels in Fig. 2*(b)* were scanned (Fig. 3*a*), the bands between basic protein and peroxidase were evident as peaks and it was possible to measure the areas under these peaks. Fig. 3*(b)* shows the progressive formation of the products at increasing concentrations of H_2O_2 . As the native basic-protein band decreased, band D_1 was formed, and, subsequently, band D_1 decreased in quantity, while band D_2 increased and material accumulated on top of the gel (Fig. 2*b*, gels 4–6, and Fig. 3*a*, arrow). Different H_2O_2 concentrations were used instead of varying the reaction times, because the time required for the proteins to enter the gels introduced an inaccuracy into time-course studies. The formation of high-molecular-weight products observed here suggests that the peroxidatic reaction forms polymers of basic protein.

Several basic proteins other than the basic protein from central-nervous-system myelin were tested for reaction with horseradish peroxidase plus H_2O_2 and remained unaffected. Lysozyme and basic protein P_2 from peripheral nervous system were incubated with 30 μ g of peroxidase plus 1.1 mM- H_2O_2 (Fig. 2*c*), and polylysine, histone and basic protein were incubated with 18 μ g of peroxidase plus

Fig. 3. Formation of new protein bands during reaction of basic protein with horseradish peroxidase plus H_2O_2 . (a) Gels shown in Fig. 2(b) were scanned. A is gel number 2; B is gel number 3; C is gel number 4. The left side is the bottom of the gel. Abbreviations: BP, myelin basic protein; HRP, horseradish peroxidase. The arrow indicates the upward deflection of the trace where stain accumulated at the top of the gel. (b) Calculated values for the areas under the peaks in gel scans from a series of incubations similar to those shown in Fig. 2(b). The areas under BP, D_1 and D_2 for each H_2O_2 concentration were added together and the area of each band was divided by the sum of the three bands. $\circ$, Native basic protein; Δ , D_1 ; $\square$, D_2 .

0.8mM- H_2O_2 (results not shown). In these 0.5h incubations all the proteins except basic protein gave their native bands on acid urea gels.

On the basis of a previous report (Sizer, 1953) that peroxidase plus H_2O_2 destroyed fibrinogen, casein and β -globulin, with concomitant changes in the u.v.-absorption spectra of the proteins and loss of tyrosyl groups, those three proteins were incubated with horseradish peroxidase plus H_2O_2 and the products examined by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (Fig. 2d). Casein (gels 6 and 7, Fig. 2d) and fibrinogen (gels 8 and 9) showed peroxidatic loss of the native protein bands, with accumulation of staining material at the tops of the gels, whereas β -globulin was unaffected (gels 10 and 11). Basic protein was incubated with lactoperoxidase plus H_2O_2 during this experiment (gels 3 and 5) and gave results similar to those obtained with basic protein plus horseradish peroxidase with H_2O_2 (gels 1 and 2). Lactoperoxidase alone produced some breakdown of basic protein (gel 4), and this effect was reproducible, suggesting a minor proteinase contamination in this enzyme preparation. Incubation with a large concentration (240 μg in 55 μl) of Sigma

horseradish peroxidase alone (results not shown) gave a small but reproducible degree of degradation of casein and fibrinogen, which is consistent with the hypothetical presence of a proteolytic contaminant in horseradish peroxidase preparations.

The peroxidatic reaction with basic protein was inhibited strongly by cyanide, azide and ferrocyanide and slightly by aminotriazole and ferricyanide (Table 1). Because dimerization of tyrosines between basic-protein molecules was the most likely hypothetical mechanism yielding polymers, as will be discussed below, tyrosine, di-iodotyrosine and phenylalanine were examined as possible competitive inhibitors (Table 1). Tyrosine and di-iodotyrosine, but not phenylalanine, gave significant inhibition at low concentrations. Catalase protected basic protein against peroxidase plus peroxide, presumably by catalysing decomposition of the H_2O_2 .

On the basis of H_2O_2 -dependence, azide-sensitivity and catalase inhibition when basic protein was attacked by small quantities of Worthington peroxidase, the reaction appeared to be peroxidatic, and the question whether basic protein was vulnerable to higher concentrations of H_2O_2 alone and/or to the 'model' system of H_2O_2 plus myoglobin (George,

Table 1. *Inhibitor effects on the disappearance of native basic protein*

Basic protein (30 μ g) was incubated with 18 μ g of Worthington horseradish peroxidase plus 0.68 mM- H_2O_2 , in the absence or presence of the inhibitors, in phosphate-buffered saline (total volume 50 μ l). After 0.5 h at room temperature (23°C), 50 μ l of 8M-urea was added to the samples, and the mixtures were electrophoresed on acid urea gels. The gels were stained, destained and scanned, and the areas under the native basic-protein bands were calculated. The data represent eight experiments and means $\pm$ S.E.M. are given where only one concentration of inhibitor was used in each of two experiments.

Compound	Concn. (mM)	Inhibition (%)
Sodium azide	1.1	65
	2.2	84
	5.5	100
	11	100
Potassium cyanide	6	100
	20	4
Aminotriazole	40	15
	60	24
Potassium ferricyanide	1.2	30 $\pm$ 10
Potassium ferrocyanide	1.0	54
	2.0	72
	3.0	78
L-Tyrosine	10	92 $\pm$ 6
DL-Phenylalanine	3.5	5
	8	19
L-Di-iodotyrosine	3.5	41
	4.0	55
Catalase (75 μ g)	—	100

1952) became of interest. A time course was measured because it was expected that the non-enzymic reactions between H_2O_2 and basic protein would be slow enough for the time of basic-protein entry into the gel to be relatively insignificant. Table 2 shows that the basic-protein band did disappear when the protein was treated with excess H_2O_2 alone. Lines 4 and 5 show, furthermore, the typical (Saunders *et al.*, 1964) inhibitor effect of excessive peroxide on peroxidase activity, since the enzyme plus 0.68 mM- H_2O_2 destroyed more basic protein in 0.5 h than did the enzyme plus 16.4 mM- H_2O_2 . The 16.4 mM- H_2O_2 , however, was more effective in the presence of peroxidase (line 4) than in its absence (line 3). Myoglobin catalysed the attack on basic protein by low concentrations of H_2O_2 . This reaction was inhibited 45% by 6 mM-sodium azide, but was not inhibited by potassium ferricyanide. Heating Worthington peroxidase or myoglobin for 2 min in a boiling-water bath inactivated their respective activities by 50 and 33%. As was found with peroxidase plus H_2O_2 (Figs. 2a and 2b), products migrating near the tops of the gels were formed when excess

Table 2. *Effects of excess H_2O_2 and of myoglobin plus H_2O_2 on native basic protein*

Basic protein (30 μ g) was incubated with horseradish peroxidase, myoglobin and/or H_2O_2 , as indicated in the Table, in phosphate-buffered saline (total volume 50 μ l). After the indicated reaction times at room temperature, 50 μ l of 8M-urea was added to the samples and the mixtures were electrophoresed on acid urea gels. The gels were stained, destained and scanned, and the areas under the native basic-protein peaks were calculated. The data represent three experiments.

[H_2O_2] (mM)	Peroxidase or myoglobin	Incubation time (h)	Basic protein degraded (%)
16.4	None	2	50
16.4	None	1	37
16.4	None	0.5	21
16.4	Peroxidase (18 μ g)	0.5	32
0.68	Peroxidase (18 μ g)	0.5	83
1.4	Peroxidase (18 μ g)	0.5	92
1.4	Myoglobin (60 μ g)	0.5	65
1.4	Myoglobin (240 μ g)	0.5	100
None	Myoglobin (60 μ g)	0.5	0
1.4	None	0.5	0

H_2O_2 alone or myoglobin plus H_2O_2 reacted with basic protein.

Discussion

When peroxidase alone (no H_2O_2) was incubated for over 1 h with basic protein (Fig. 1d) and basic protein was degraded, the absence of inhibition by cyanide and fluoride (Keilin & Mann, 1937) and only very slight inhibition by catalase and azide suggested that the mechanism of destruction of basic protein was not predominantly peroxidatic. The lability, with respect to basic-protein breakdown, of peroxidase preparations to heat, the formation from basic protein of low-molecular-weight breakdown products (Johnson & Cammer, 1977), and the various activities for basic-protein degradation in peroxidase preparations from different sources all suggested a proteolytic enzyme contaminating the peroxidase preparations. Although basic-protein degradation was initially observed with Sigma type VI peroxidase, which is predominantly the basic isoenzyme, the acidic isoenzyme preparations (types VII and VIII) also attacked basic protein, suggesting that a proteolytic contaminant was present in all these peroxidase samples. The outstanding vulnerability of the myelin basic protein to proteolytic attack is a well-known phenomenon (e.g. Eylar, 1973), and the absence of degradation of most of the other proteins tested underlines the difficulty expected if synthetic substrates for proteinases are to be used along with peroxidase preparations in attempts to demonstrate traces of a proteinase contaminant. The partial

degradation of casein and fibrinogen during a 4 h incubation with 240 μ g of Sigma peroxidase and no H_2O_2 (results not shown) supports the conclusion that a proteinase is present in the peroxidase preparations.

Although the effect of high peroxidase concentrations alone on basic protein has practical implications for immunohistochemists (Johnson & Cammer, 1977), the reaction of catalytic amounts of the enzyme plus H_2O_2 with basic protein to form high-molecular-weight products is of greater fundamental importance. Inhibition by azide, cyanide and catalase (Table 1) and the effects of the peroxidase and H_2O_2 concentrations on the extent of disappearance of the basic-protein band (Figs. 2a and 2b) indicate horseradish peroxidase catalysis of a peroxidatic reaction with basic protein (Keilin & Mann, 1937). Ferricyanide and ferrocyanide would inhibit by either binding to the haem iron in the enzyme or interfering with the sequence of oxidation and reduction reactions involved in the peroxidatic effects on basic protein. One mechanism by which peroxidase reacts with proteins is halogenation, primarily utilizing iodide as well as H_2O_2 (Morrison & Schonbaum, 1976). This is not likely to be the mechanism for the reaction of peroxidase with basic protein plus H_2O_2 , because iodide was never included in our reaction medium, and chloride, although usually present, was also not required. In a report stating that basic protein was degraded by ascorbic acid or H_2O_2 (Westall *et al.*, 1976), a Figure showed a gel pattern of basic protein that had been incubated with 10mM- $FeCl_3$ and 10mM- H_2O_2 , where the products formed has higher molecular weights than that of the native basic protein. That reaction is probably similar to the peroxidatic reaction reported here.

The peroxidatic dimerization of the phenol groups of cresol has been described (Hewson & Dunford, 1976), and a similar mechanism could utilize any of the four tyrosine phenolic groups (Eylar, 1973) in the basic-protein molecule. Previous observations (Sizer, 1953) describing spectral evidence of changes in the tyrosine groups of proteins during peroxidatic reaction also suggested this route for polymerization. During our attempts to devise time-course studies for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of basic-protein products, we tried to stop the polymerization reaction by using azide, which, when present at the outset, inhibits the peroxidatic reaction (Table 1). However, once begun, the formation of high-molecular-weight products could not be stopped by azide, suggesting an initial peroxidatic formation of tyrosyl free radicals, followed by spontaneous dimerization of these groups, ultimately linking many chains of basic protein. When the initial reaction was slowed down by using suboptimal concentrations of H_2O_2 , and the

reaction mixtures were electrophoresed on acid urea gels, in which the products and intermediates are rapidly separated (Johnson & Cammer, 1977), progressive formation of products with increasing molecular weights was demonstrated (Fig. 2b). This phenomenon was not so apparent when we used the sodium dodecyl sulphate/polyacrylamide-gel system, because the initial dimer, with an expected mol.wt. of 36000, was obscured by the peroxidase band (mol.wt. 40000) on 15% polyacrylamide gels, and on sodium dodecyl sulphate/polyacrylamide gels containing lower quantities of acrylamide, the native basic-protein band (mol.wt. 18000) moved with the dye front. In the acid urea gels (Fig. 2) the basic-protein products migrated ahead of the peroxidase because of their highly basic nature. Inhibition by tyrosine and di-iodotyrosine (Table 1) gives further support to a mechanism involving dimerization of phenol groups as a route for forming high-molecular-weight products from basic protein via linkage between tyrosine moieties in different basic-protein chains.

The physiological significance of this reaction is not yet apparent. The resistance of other basic proteins, namely, lysozyme, P_2 protein and histone, shows that, in addition to being outstandingly vulnerable to proteolytic attack (Eylar, 1973), the myelin basic protein is also unusually susceptible to peroxidatic reactions involving either horseradish peroxidase plus H_2O_2 , such 'model' systems as myoglobin plus H_2O_2 , excess H_2O_2 alone, or lactoperoxidase plus H_2O_2 . It was not surprising that horseradish peroxidase preparations attacked basic protein, fibrinogen and casein by both the proteolytic and the peroxidatic mechanisms, whereas P_2 protein, lysozyme and histone were not attacked by either mechanism. Whereas all six of these proteins contain tyrosine (Dayhoff & Eck, 1973; Braun & Brostoff, 1977), which would be a necessary condition for vulnerability to the postulated peroxidatic mechanism, basic protein, fibrinogen and casein apparently all possess regions lacking in secondary structure, a property that would both make their tyrosine moieties vulnerable to peroxidatic attack and render regions of their amino acid chains available for proteolytic digestion. It is relevant that, in addition to fibrin, casein (Christensen, 1945) and basic protein (Cammer *et al.*, 1977) both serve as substrates for plasmin, which is a relatively specific proteolytic enzyme.

The peroxidatic reaction with basic protein may have physiological consequences under conditions where myelin is disrupted, such as an acute inflammatory response in which basic protein can be exposed to peroxidases in brain cells or invading mononuclear cells in the presence of the increased H_2O_2 concentrations occurring during phagocytosis, or even the H_2O_2 arising normally during tissue res-

piration. There is considerable endogenous peroxidase activity in the brain parenchyma as revealed by diaminobenzidine staining (Hruban *et al.*, 1972; de Duve, 1973; Keefer & Christ, 1976). There is a possibility that this staining is due to catalase, since it is inhibited by aminotriazole. However, we find that aminotriazole also inhibits horseradish peroxidase (Table 1). Biochemical studies have shown true peroxidase activity, clearly distinguished from catalase, in many brain areas (Van Woert & Ambani, 1974). Abnormal conditions allowing access of cellular enzymes to basic protein in myelin would facilitate catalysis of peroxidatic attack on this protein.

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