Regular Fragmentation of Hydrogen Peroxide-treated Fibronectin*

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In the presence of low concentrations (<0.5 mM) of hydrogen peroxide M, 350,000 and 170,000 fragments were generated from plasma and fibroblast medium fibronectins (Fns). No other major fragments were detected when H$_2$O$_2$ concentration was raised or the incubation time prolonged. A 200–300-fold concentration of H$_2$O$_2$ was needed for a complete degradation of the protein. The degradation was inhibited or completely prevented by deferoxamine, diethylene-triaminepentaacetic acid, and thiourea or by Chelex-pretreatment of the Fn solution suggesting a Fenton-type reaction to produce *OH radicals from H$_2$O$_2$. In immunoblotting the M, 170,000 fragment reacted with monoclonal antibodies against the NH$_2$ terminus and mid-molecule but not with those against the cell-binding site and the COOH terminus of M, 350,000 fragment. Reduction of the M, 350,000 fragment produced α- and β-monomers of Fn as well as M, 95,000 and 85,000 fragments which reacted with monoclonal antibodies against the cell-binding site and the COOH terminus of Fn. These results suggest that the M, 170,000 fragment is derived from the NH$_2$-terminal part of both subunits of Fn. The rest of the subunits, the M, 95,000 (from α-chain) and M, 85,000 (from β-chain), thus remain disulfide-bonded to an intact Fn subunit to form the nonreduced M, 350,000 polypeptide. The results show that oxygen radical action may generate defined and reproducible fragments from Fn. The high susceptibility of Fn to the radical induced degradation makes it plausible to occur also in vivo.

Fibronectin (Fn) is a high molecular weight glycoprotein that exists in soluble form in plasma and other body fluids and in insoluble form in connective tissues (for reviews, see Refs. 10–15). The protein is composed of two disulfide-bonded subunits, interacts with several kinds of molecules, and mediates cell adhesion. The wide distribution of Fn makes it a plausible object to encounter various destructive agents. Therefore it may also be a target for action of oxygen-derived free radicals. In the present paper the effect of H$_2$O$_2$ on Fn was investigated. In vivo the respiratory burst of phagocytic cells is a major source of H$_2$O$_2$ that itself is quite inactive. In the presence of transition metal ions it, however, is reduced to form hydroxide ion (OH$^-$) and hydroxyl radical. The latter is the most active of the oxygen-free radicals and can destroy other molecules. The results show that in the presence of H$_2$O$_2$, Fn is degraded in a highly regular manner to produce defined fragments.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Fn was purified from human plasma and horse serum (Gibco, Paisley, Scotland) by affinity chromatography on gelatin-Sepharose (Pharmacia, Uppsala, Sweden) as described (16). Purified Fn was disulfylated in PBS (0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.4). Protein concentrations were measured according to Lowry et al. (17) using bovine serum albumin as a standard.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (18), using vertical slab gels. The acrylamide concentration was 3.5% in the spacer gel and 6% in the separating gel. The samples were run nonreduced or reduced with 10% 2-mercaptoethanol in Laemmli sample buffer as indicated. After electrophoresis, the gels were either stained with Coomassie Brilliant Blue (19), fluorographed (20), or subjected to immunoblotting (see below).

**Immunoblotting**—Immunoblotting was performed as described by Towbin et al. (21). In this procedure polypeptides separated by SDS-PAGE were transferred electrophoretically to nitrocellulose sheets (type I HAWP filter, Millipore, Bedford, MA). The polyclonal rabbit antiserum against human plasma Fn and monoclonal antibody against the NH$_2$ terminus of the protein were as described (22, 23). Monoclonal antibodies against the mid-molecule (N293, IgG$_1$), cell-binding site (N295, IgG$_2$), and COOH terminus (N296, IgG$_3$) of Fn were from Mannikcrodt (St. Louis, MO). Immunoreactions were detected by using peroxidase-coupled rabbit anti-mouse or swine anti-rabbit IgG antiserum (Dakopatts, Glostrup, Denmark).

**Experiments with H$_2$O$_2$**—The experiments were carried out essentially as in some previous publications (24–27). PBS was employed since phosphate is present in vivo and since many other buffers (e.g., Tris and Hepes) react with *OH radicals (28, 29). To better simulate possible in vivo conditions, traces of metal ions were not removed from PBS in the usual experiments. Hydrogen peroxide (Merck) was obtained as a 30% solution from Merck (Darmstadt, Federal Republic of Germany) and added in the Fn solutions to give final concentrations of 0.5–200 mM. The reaction mixtures were then incubated at 37 °C in the presence of H$_2$O$_2$ for various periods of time.

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*The abbreviations used are: Fn, fibronectin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; TLCK, N-$p$-tosyl-L-$p$-lysine chloromethyl ketone.
as indicated. In some experiments, catalase (from bovine liver, thymol-free 11,000 units/mg; Sigma), deferoxamine mesylate (a gift from Ciba-Geigy, Basel, Switzerland), diethylenetriaminepentaacetic acid (Sigma), or thiourea (Sigma) were added into the reaction mixtures before H$_2$O$_2$. After the incubations, the samples (volumes 10–30 μl in all experiments) were mixed in Laemmli sample buffer containing 0.3 mM deferoxamine and the whole reaction mixtures were analyzed in SDS-PAGE under nonreducing conditions. In some experiments the Fn solution was passed through a column of Chelex-100 resin (Bio-Rad). Then H$_2$O$_2$ was added as above in the presence or absence of FeSO$_4$·7H$_2$O (Merck).

In a set of experiments, Fn was incubated with H$_2$O$_2$ in the presence of methylenebis (4-aminoethyl) ether hydrochloride (Sigma), concentration 1 mM, soybean trypsin inhibitor (20 μg/ml), N-acetyl-L-lysine (TLCK, 20 μg/ml), N-ethylmaleimide (5 mM), iodoacetamide (5 mM), or pepstatin A (5 μg/ml). All the reaction mixtures were then mixed with Laemmli sample buffer containing 0.3 mM deferoxamine for analysis in SDS-PAGE.

**Cell Culture**—Human embryonic skin fibroblasts were established locally. The cells were grown at 37 °C in a humidified 5% CO$_2$ atmosphere in Eagle’s minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories) and antibiotics. For labeling with [~S]methionine (20 pCi/ml) supplemented with 0.2% bovine serum albumin. After incubation of 8 h, the medium was collected and clarified by centrifugation. Proteins in 0.5-ml aliquots of the medium were then treated with 40 μl of 50% (v/v) gelatin-Sepharose 4B or NaCl-containing Sepharose 4B, Sepharose CL-4B, and Sepharose 4B were obtained from Pharmacia LKB Biotechnology Inc. Spermine tetrahydrochloride (Serva, Heidelberg, Federal Republic of Germany) was coupled to activated CH-Sepharose 4B according to the instructions of the manufacturer. Unconjugated Sepharoses were used in control experiments.

Relative Quantifications of Protein Bands in SDS-PAGE Gels—Aliquots of Fn (8 μg) were treated with increasing concentrations of H$_2$O$_2$ at 37 °C for 1 h and then analyzed in SDS-PAGE under nonnondenaturing conditions as described above. The protein-stained gels were photographed and the negatives were enlarged on Kodak RP X-Omat x-ray films to get the positive images resembling usual autoradiographs. The developed films were then scanned by a densitometer and relative quantities of the polypeptide bands were estimated from the scanning profiles.

**Other Methods**—Heparin-Sepharose 4B, CNBr-activated CH-Sepharose CL-4B, and Sepharose 4B were obtained from Pharmacia LKB Biotechnology Inc. Spermine tetrahydrochloride (Serva, Heidelberg, Federal Republic of Germany) was coupled to activated CH-Sepharose 4B according to the instructions of the manufacturer. Unconjugated Sepharoses were used in control experiments.

Multiple parallel aliquots of Fn (8 μg) were incubated with increasing concentrations of H$_2$O$_2$ for various periods of time at 37 °C. The reactions were stopped by addition of 0.3 mM deferoxamine and one of each parallel reaction mixture was analyzed in SDS-PAGE under nonreducing conditions, and the remaining materials were stored at -20 °C. After the electrophoretic analysis, the stored samples corresponding to those that in protein-stained gel contained seemingly dimeric Fn as well as the corresponding to those that in protein-stained gel contained seemingly monomeric proteins at 350,000, 170,000, and 70,000 protein bands were excised from the gel. The gels slices were then equilibrated in reduced Laemmli sample buffer, applied into preformed slots in a spacer gel of another SDS-PAGE, which was then run and subjected to immunoblotting. In some experiments SDS was removed from the excised gel slices by incubating in 1 ml of 2.5% Triton X-100 (v/v) in PBS for 2 × 20 min and then in PBS for another 2 × 20 min. The slices were then treated with various concentrations of H$_2$O$_2$, equilibrated in nonreduced Laemmli sample buffer containing 0.3 mM deferoxamine, and rerun in SDS-PAGE for immunoblotting.

**RESULTS**

**Degradation of Fn in the Presence of H$_2$O$_2$—** Fn was incubated in the presence of H$_2$O$_2$ and analyzed in SDS-PAGE under nonreducing conditions. Nontreated Fn (8 μg) showed a prominent dimeric polypeptide band and a group of apparently monomeric proteins at M, 200,000–270,000 (Fig. 1A, lane 1). Incubation with 0.5–80 mM H$_2$O$_2$ produced two additional polypeptides, M, 170,000 and 350,000, and the dimeric Fn was almost completely degraded at a concentration of 200 mM H$_2$O$_2$ (Fig. 1A; 5 min incubation). The digestion pattern was essentially similar after 30 min incubation (Fig. 1B), but later more complete degradation occurred also at lower concentrations of H$_2$O$_2$ and 200 mM H$_2$O$_2$ degraded all polypeptides (Fig. 1C; 2 h incubation). Then also other fragments were generated (Fig. 1C, lane 3), whereas at lower concentrations even 12% gels did not reveal fragments other than M, 170,000 and 350,000. The appearance of M, 170,000 and 350,000 fragments could be detected in immunoblotting by anti-Fn antibodies even at H$_2$O$_2$ concentrations as low as 10–100 μM (not shown). Aliquots of 2 μg of Fn were treated with increasing concentrations of H$_2$O$_2$ for various periods of time. The reaction mixtures were then analyzed in SDS-PAGE under nonreducing conditions and the time points of disappearance of dimeric Fn were recorded to obtain an average dose/time relationship curve for complete degradation of Fn (Fig. 2). Fig. 3 shows the disappearance of dimeric Fn versus the appearances of the M, 170,000 and 350,000 fragments as a function of H$_2$O$_2$ concentration. It can be seen that while both fragments appear simultaneously, the M, 170,000 is a bit more resistant for further proteolysis than the M, 350,000 fragment.

Horse serum Fn (Fig. 4A) and cell culture medium Fn (Fig. 4B) were similarly degraded in the presence of H$_2$O$_2$. The gelatin-binding M, 70,000 protein (30) was instead less affected (Fig. 4B). The intact dimeric Fn as well as the M, 170,000 and 350,000 polypeptides bound to gelatin-, spamine-, and heparin-Sepharoses (not shown). All bound proteins were eluted from gelatin-Sepharose by urea concentrations exceeding 4 M, from spermine-Sepharose by a 200 mM NaCl concentration, and from heparin-Sepharose with NaCl above 250 mM. No differences were found when Sepharose binding or elution of H$_2$O$_2$-treated or similar amounts of

![Fig. 1. Incubation of human plasma Fn in the presence of H$_2$O$_2$. Human plasma Fn (8 μg) was incubated in the presence of 0.5 (lanes 2), 40 (lanes 3), or 200 mM (lanes 4) H$_2$O$_2$ for 5 min (A), 30 min (B), or 2 h (C) at 37 °C. Lanes 1 show control Fn incubated similarly in the absence of H$_2$O$_2$. After incubations, the samples were run nonreduced in a 6% SDS-PAGE. dFn, dimeric Fn.](image-url)
(ranging between 2 and 100 mM) of H₂O₂ for various periods of time (10 min to 14 h) at 37 °C. After the incubations the samples were analyzed in SDS-PAGE under nonreducing conditions. For each H₂O₂ concentration used, the time points of total degradation of dimeric Fn were recorded from protein-stained incubations the samples were analyzed in SDS-PAGE that had been scanned by a densitometer to their maximum signal assigned to have a value of 100%.

FIG. 2. Dose/time dependence of the degradation of Fn. Aliquots of human plasma Fn (2 μg each) were incubated in the presence of various concentrations (ranging between 2 and 100 mM) of H₂O₂ for various periods of time (10 min to 14 h) at 37 °C. After the incubations the samples were analyzed in SDS-PAGE under nonreducing conditions. For each H₂O₂ concentration used, the time points of total degradation of dimeric Fn were recorded from protein-stained gels for the dose/time dependence curve.

FIG. 3. Kinetics of the degradation of dimeric Fn and the formation of the 170,000 and 350,000 fragments. Human plasma Fn (8 μg) was treated with increasing concentrations of H₂O₂ and analyzed in SDS-PAGE that had been scanned by a densitometer as described under "Experimental Procedures." The amounts of dimeric Fn, M, 170,000 (A), and 350,000 (B) fragments were related to their maximum signal assigned to have a value of 100%.

nontreated Fn were compared. Unconjugated control Sepharoses bound only negligible amounts of proteins.

Inhibition and Activation Experiments—Degradation of Fn was markedly inhibited if the protein solution was pretreated with Chelex or if 0.3–0.8 mM deferoxamine was added before H₂O₂ in the reaction mixtures (Fig. 5). In a similar analysis diethylenetriaminepentaacetic acid (0.1–1 mM tested), catalase (1–10 μg/ml), and thiourea (5–50 μM), also inhibited or completely prevented the degradation of Fn (not shown). On the other hand, adding of FeSO₄ (1–5 mM) into the Chelex-treated Fn solutions enhanced the degradation and the production of the M, 170,000 and 350,000 polypeptides in the presence of H₂O₂ (not shown). The presence of protease inhibitors, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, TLCK, N-ethylmaleimide, iodoacetamide, or pepstatin did not prevent the H₂O₂-induced degradation of Fn.

Immunoblotting—Nonreduced M, 170,000 and 350,000 polypeptide bands were excised from SDS-PAGE gels, reduced, and proceeded to immunoblotting by monoclonal Fn antibodies as described under "Experimental Procedures." Under reducing conditions the M, 170,000 polypeptide migrated slightly slower (to approximately M₁, 172,000) and in immunoblotting reacted with monoclonal antibodies against the NH₂ terminus and mid-molecule of Fn (Fig. 6B, lanes 1 and 2), but not with those against the cell-binding site and the COOH terminus (Fig. 6B, lanes 3 and 4). Reduction of the M, 350,000 polypeptide produced Fn monomers and two additional polypeptide bands: M, 95,000 and 85,000 (Fig. 6C, lanes 3 and 4). The monomeric Fn reacted with all of the above-mentioned monoclonal antibodies (Fig. 6C, lanes 1–4) while the M, 95,000 and 85,000 polypeptides were detected only with the antibodies against the cell-binding site and COOH terminus of Fn (Fig. 6C, lanes 3 and 4). To better demonstrate the reduced α- and β-chains of Fn, the M, 350,000 fragment of H₂O₂-treated horse serum Fn was excised, reduced, re-electrophoresed, and immunoblotted by the monoclonal antibody against the NH₂ terminus of the protein (Fig. 6C, lane 5). Fig. 6A, lane 1, shows nonreduced Fn, and lane 2 H₂O₂-treated (0.5 mM) Fn immunoblotted with polyclonal rabbit anti-Fn antibodies. When excised gel slices of dimeric Fn were treated with various concentrations of H₂O₂, rerun in another gel, and immunoblotted with polyclonal rabbit anti-Fn antibodies, a group of high molecular weight fragments were generated (Fig. 6D). One of these fragments was
the $M_s$ 170,000 while the $M_s$ 350,000 fragment could not be positively identified. The appearance of a group of higher molecular weight fragments (even in the nontreated sample, Fig. 6D, lane 1) may have been due to altered experimental conditions compared with Fn in solution.

**DISCUSSION**

The present results shown that Fn is readily degraded in a highly regular pattern of fragments in the presence of small or moderate concentrations of $H_2O_2$. Considerably higher amounts of $H_2O_2$ were needed to obtain total degradation of the protein. Fragmentation was similar when Fns from different sources were treated with $H_2O_2$. Even immobilizing fibroblast medium Fn to gelatin-Sepharose did not change the reaction. In the experiments the whole reaction mixtures (including those containing Sepharose-beads) were applied into the gels in SDS-containing sample buffer. Thus, the gradual loss of protein (e.g. in Figs. 1 and 4) is indeed due to degradation and not to precipitation or adherence of Fn to the walls of the reaction vessels or Sepharose beads.

Inhibitory effect of deferoxamine, diethylentriaminepentaacetic acid, catalase, and thiourea emphasize the role of $\cdot$OH radicals in the degradation. Most probably the trace amounts of iron ions present in practically all laboratory autums of iron ions present in practically all laboratory buffers participate in the Fenton-type reaction:

$$Fe^{++} + H_2O_2 \rightarrow Fe^{++} + \cdot OH + OH^-$$

**Fig. 6. Immunoblotting of Fn and its reduced fragments.**

Human plasma Fn was treated with 0.5 mM $H_2O_2$, electrophoresed nonreduced in SDS-PAGE which was protein stained under nonfixing conditions. Stained $M_s$ 170,000 and 350,000 polypeptide bands were excised from the gel, equilibrated in reduced Laemmli sample buffer, and rerun in another 6% SDS-PAGE which was then immunoblotted. A: lane 1, immunoblotting of nonreduced Fn; lane 2, $H_2O_2$ (0.5 mM)-treated Fn with a polyclonal rabbit Fn antiserum. Immunoblotting of reduced $M_s$ 170,000 (B) and reduced $M_s$ 350,000 (C) polypeptides with a monoclonal antibody against the NH$_2$ terminus of Fn (lanes 2), against the mid-molecule of Fn (lanes 2), against the cell-binding site of Fn (lanes 3), and against the COOH terminus of Fn (lanes 4). Lane 5 (C) shows similarly treated $M_s$ 350,000 fragment of horse serum Fn immunoblotted by the monoclonal antibody against the NH$_2$ terminus of Fn. D, immunoblotting of a re-electrophoresed gel slice of nonreduced Fn (lane 1) and that treated with 0.5 mM (lane 2) or with 40 mM (lane 3) $H_2O_2$.

**Fig. 7. Schematic presentation of the cleavage of Fn by $H_2O_2$.** The thin arrows indicate the suggested cleavage sites in intact dimer Fn. The arrowheads indicate the sites of determinants for the monoclonal antibodies: 1, the NH$_2$ terminus; 2, mid-molecule; 3, cell-binding site; and 4, the COOH-terminus of Fn.

The physiological concentrations of non-protein-bound iron salts are a few micromolar (7), which may also be present in PBS used in the experiments. In line with this, pretreating of Fn solutions with Chelex reduced or completely prevented the degradation of Fn. On the other hand, adding of Fe$^{++}$ ions to Chelex-treated Fn solution induced the degradation under the same conditions. Furthermore, essentially similar degradation pattern was obtained when excised gel slices containing dimeric Fn were treated with $H_2O_2$. This suggests a direct effect of $\cdot OH$ radicals on Fn degradation rather than a structural modification of the protein which could have made it more susceptible to fragmentation caused by a possible contaminant protease in the original Fn preparations. The latter possibility was also excluded by the fact that none of the various protease inhibitors tested prevented the degradation of Fn in the presence of $H_2O_2$.

Treatment of Fn with $H_2O_2$ produced two major fragments, $M_s$ 170,000 and 350,000. These polypeptides retained at least some of the activities of Fn such as an ability to bind gelatin, spermine, and heparin and no detectable differences were found in elution of intact dimeric Fn and the $M_s$ 350,000 and 170,000 fragments from Sepharose. Binding properties and immunoblotting results indicate that the $M_s$ 170,000 fragment is cleaved from the NH$_2$-terminal part of Fn. Thus it is an unusually large NH$_2$-terminal fragment but still does not contain the cell-binding site of Fn. Since only one polypeptide of this size was detected, it is presumed that it is cleaved at the same distance from the NH$_2$-termini of both $\alpha$- and $\beta$-chains of Fn. This assumption is based on the fact that the reduction of the $M_s$ 350,000 protein generated Fn monomers as well as the $M_s$ 95,000 and 85,000 fragments, which contained COOH-terminal domains of Fn. The different sizes suggest an origin from either $\alpha$- (the $M_s$ 95,000 fragment) or $\beta$- (the $M_s$ 85,000 fragment) subunits of Fn. These conclusions are consistent with the scheme presented in Fig. 7. Thus, the nonreduced $M_s$ 350,000 fragment is actually composed of two disulfide-bonded polypeptides: $\alpha$-chain + $M_s$ 85,000 fragment and $\beta$-chain + $M_s$ 95,000 fragment. The molecular weights of both of these combinations are equal ($M_s$ 350,000). According to this scheme the molecular weight of $\alpha$-chain would be 265,000 (170,000 + 95,000) and that of $\beta$-chain 250,000 (170,000 + 85,000) and hence Fn-dimer would be 520,000.

The reason for the apparent alternative cleavage of the subunits of Fn is not known. It may, however, be that after the primary cleavage of one of the subunits, the remaining intact chain of the dimer may undergo a new more protective conformation. The fact that any of the amino acids may be susceptible to free hydroxyl radical action (31) further suggests an appropriate conformation for cleavage in case of Fn. Furthermore, an involvement of some particular amino acid(s) or peptide bond(s) in the degradation would most probably cause multiple cleavage sites instead of the one detected in the present study.
Oxidative processes may affect Fn in several ways. Apart from the degradation presented here, considerably smaller quantities of oxygen-derived radicals may release intact tissue from its surroundings as shown by Peters et al. (32). On the other hand, higher concentrations of transition metals in the absence of oxygen radicals may induce polymerization of Fn (33). Thus, it might be hypothetized that in oxidant-induced tissue injury, Fn may become disorganized or degraded while different oxidative mechanisms may enhance the contribution of the protein in tissue repair.

Previous studies have shown that at least in some occasions the free radical induced degradation of proteins generates defined fragments (26, 34, 35). Also in the case of Fn, the degradation seems to be highly regulated. This is especially true at concentrations of H2O2 as low as to be expected to occur in vivo. In some previous reports activated phagocytes in vitro have been shown to produce concentrations of a few nanomoles to hundreds of micromoles of H2O2 depending on the number of cells or detection times in the experiments (36–38). Thus, the lowest H2O2 concentrations used in the present study may occur in vivo, although those exceeding 0.5 mM are most probably unlikely. Oxygen-derived free radicals in various pathologic situations (see Introduction) in vivo do not usually diffuse long distances (6), which may produce also moderately high local concentrations. Whatever the case may be, H2O2 and traces of non-protein-bound iron salts are present in vivo (for review, see Ref. 7) and could produce ·OH radicals. These are the major species responsible for oxygen toxicity while H2O2 itself or the superoxide radical are far less active. When highly reactive hydroxyl radicals are formed in living tissues, they will react immediately with whatever biological molecule is in their vicinity. Fn, due to its fairly ubiquitous distribution, may be present in many of the ·OH radical generating pathologic situations and may hence be among the targets of their destructive action.

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