Amino Acids (1992) 3: 183–194

Amino
Acids

Oxygen radical induced fluorescence in proteins; identification of the fluorescent tryptophan metabolite, N-formyl kynurenine, as a biological index of radical damage

H. R. Griffiths,[1] J. Lunec[1], and D. R. Blake[2]

[1] Molecular Toxicity Group, University of Leicester, Glenfield Hospital, Leicester, and
[2] Bone and Joint Research Unit, London Hospital Medical College, London, England

Accepted November 15, 1991

Summary. The effect of oxygen derived free radicals (OFR) on aromatic and sulphur containing amino acids has been investigated, both in their free form and within protein backbones. Aerated amino acids and proteins in solution were exposed to three discrete OFR generating systems; (1) gamma radiation in the presence or absence of formate (2) photolysis by UV light at 254 and 366 nm, and (3) site specific modification by H_2O_2 in the presence of CuII ions.

A sensitive reverse-phase HPLC technique with dual detection systems (UV absorbance and fluorescence monitoring) was developed to analyse the products of amino acid oxidation. OFR denatured amino acids were chromatographed by this procedure, and all radical species generated, with the exception of the superoxide anion, resulted in the formation of identifiable fluorescent metabolites of tryptophan, kynurenines. The identity of peaks was confimed by spiking with authentic material and scanning absorption spectroscopy. After complete proteolytic hydrolysis, OFR treated proteins were also analysed by this technique; again the dose dependent production of kynurenines was detected in IgG, γ lens crystallins and albumin. Bityrosine was not detected in any of the proteins studied using this procedure, however, several novel unidentified fluorophores were detected in proteolytic hydrolysates, possibly the product of two different amino acid radicals.

Immunoglobulin G isolated from the sera of normals and rheumatoid arthritis (RA) patients was examined for the presence of one specific tryptophan metabolite, N-formyl kynurenine. Significantly elevated levels of this metabolite were detected in rheumatoid sera, suggesting increased OFR activity in RA.

These results have demonstrated firstly, that specific oxidised products of amino acids are retained in the protein backbone after exposure to OFR generating systems. Secondly, in aerated solution, oxidised tryptophan residues confer the major new visible fluorescence in non-haem proteins, not tyrosine

products. In addition, this work has demonstrated that the measurement of a specific product of an oxidised amino acid can be applied to biological macromolecules, and may be important in implicating free radical reactions in certain disease processes.

Keywords: Amino Acids – Protein fluorescence – Kynurenines – Oxygen free radicals

Introduction

The oxidation of biomolecules appears to be a normal consequence of biological aging and evidence is accumulating to suggest that this process may be accelerated in certain disease states [1, 2]. The most well-documented of these pathologies include rheumatoid arthritis [3, 4], adult respiratory distress syndrome [5] and cataract lens formation [6], where oxidative damage to the proteins IgG [7, 8], α-1-antitrypsin [9] and γ lens crystallins [10] have been described. It has been postulated that the nonspecific oxidation of biomolecules in these pathologies proceeds via oxygen free radical (OFR) intermediates. These chemical species are highly reactive with nearly all known biomolecules, having reaction rates in the order of 10^9M/sec [11].

The induction of lipid peroxidation by OFRs is well documented [12], however, only recently have the effects of OFR on proteins been studied in detail [10, 13 and 14]; because of their functional diversity, any chemical modification may be expected to influence many biological processes.

The exposure of proteins to OFR in vitro has been shown to induce aggregation [6, 15], fragmentation [16], carbonyl formation [17, 18] and the generation of autofluorescence in the visible region (Excitation max = 360 nm, Emission max = 454 nm) [19]; the nature of the effects depending on the oxidising species, the protein under study and its environment. Similar changes have also been described in proteins isolated from diseased tissues. For example, both gamma lens crystallins isolated from cataractous lenses and IgG from the synovial fluids of rheumatoid patients are found as aggregates and exhibit a non-tryptophan autofluorescence in the visible region [20, 8]. Previously, these crude measurements of carbonyl formation and fluorescence generation have been used to monitor OFR attack in vivo, however, both measures are subject to interference from bound lipids and particularly in haem proteins, from bound metal ions.

It is likely that the macromolecular changes observed following protein oxidation are a manifestation of modifications at the molecular level to specific amino acids. The most susceptible residues are the aromatic amino acids (where stabilisation of radical intermediates can be achieved by the delocalisation of unpaired electrons around the ring) and sulphur containing amino acids (where increasing the valency of the sulphur moiety allows stabilisation of radical intermediates) [21, 22]. The isolation and identification of tryptophan photoproducts has been extensively undertaken by Singh et al, where the expected yields of kynurenines and the intermediates in their formation following OFR

attack have been described [23]. Studies on the irradiation of poly-L-tyrosine led to the identification of bityrosine, a fluorescent product (Excitation maximum = 290 nm, emission maximum = 410 nm) [24]. It has been proposed that the formation of bityrosine from two spatially adjacent tyrosine residues may confer the new visible fluorescence induced in an oxidized protein [14, 25]. However, other workers have disputed this and claim that tryptophan oxidation with the concomitant production of kynurenines may account for the major part of the fluorescence formation [26, 27]. Since neither metabolite would be incorporated into a protein backbone during synthesis, the presence of either is indicative of damage by an OFR dependent mechanism.

In order to unequivocally state that an oxygen radical mechanism is responsible for the oxidative changes to proteins which occur during aging or disease, the presence of specific modified residues must be demonstrated within the backbone of isolated proteins. We have therefore developed a reverse phase HPLC technique to examine the effects of OFR attack on proteins in vitro. Herein we describe the effects of OFRs on the component aromatic and sulphur containing amino acids, the formation of specific fluorescent products of tryptophan oxidation (namely kynurenines) but not tyrosine oxidation and the detection of these products in proteins oxidised in vitro, and in IgG isolated from pathological biofluids.

Methods

Materials

Immunoglobulin G, pepsin, γ lens crystallins, amino acids, hydrogen peroxide and pronase E were all obtained from Sigma, Poole, Dorset, UK. The reverse phase C18 ODS2 (5 μm) column used for HPLC was obtained from Technicol, Macclefield, Cheshire. HPLC grade acetonitrile was purchased from May and Baker, UK. All other reagents were of Analar grade from BDH, Poole, Dorset, UK. Clinical specimens were obtained from patients attending clinics at Selly Oak Hospital, Birmingham or from the Blood Transfusion Service, The Medical School, University of Birmingham, UK.

Oxygen radical generation

Steady state radiolysis was used as a tool to generate specific radicals in solution. Briefly, proteins and native amino acids were made up to 1 mg/ml in 40 mM phosphate buffer pH 7.2. Aerated solutions were then exposed 0, 250,500 and 1000 gy irradiation doses at a dose rate of 10 gy per minute from a ^{60}Co gamma source, generating hydroxyl ($^{\bullet}$OH) and superoxide anion radicals ($O_2^{\bullet -}$) in a ratio of 2 : 1.

To other experimental solutions, the addition of 0.2 M formate to scavenge $^{\bullet}$OH yielded only $O_2^{\bullet -}$ in solution.

As a model system for metal ion catalysed site specific modification, 20 μM CuII as copper sulphate was added to proteins (1 mg/ml in PBS) prior to the addition of 0, 20, 50 or 200 μM H_2O_2. The presence of CuII alone did not induce any detectable changes in the proteins under study.

Since much work has been done on the photolytic decomposition of amino acids, UV irradiation was also used as a model denaturation system. The UV source (from Andermann and Company Ltd, Surrey) was placed 6 cm away from the sample and emitted light at wavelengths of 366 and 254 nm. This corresponds to average light intensities of 17 uW/cm^2 and 10.5 uW/cm^2 respectively at 1 m, according to the manufacturer's specifications.

Isolation of immunoglobulin G from biological fluids

To examine the effects of accelerated aging in vivo on constituent amino acids, immunoglobulin G was isolated from the sera and synovial fluids (SFs) of patients with rheumatoid arthritis and normal control sera. After 24 hour treatment with hyaluronidase to hydrolyse hyaluronic acid present in SFs, a combination of caprylic acid precipitation and selective salt precipitation with 40% ammonium sulphate was used to isolate IgG according to the method of McKinney et al [28]. Immunoelectrophoresis of the isolated protein against anti-human serum resulted in one single precipitation band corresponding to IgG, indicating a pure preparation. Protein determination was according to the method of Lowry [29] and IgG was adjusted to a final concentration of 1 mg/ml in PBS.

Protein digestion

To hydrolyse the denatured proteins to their constituent amino acids, enzymic digestion was performed using pronase E at 37°C, the final concentration ratio being 1 mg/ml pronase: 20 mg/ml protein. This was carried out in the presence of 50 μM mannitol as an antioxidant and 100 μg/ml streptomycin sulphate as a bacteriostat. Under these conditions hydrolysis was complete within 20 hours.

HPLC

An apple IIe microprocessor controlled Gilson Liquid Chromotograph equipped with a variable wavelength detector and fluorimeter (excitation range 305–395 nm, emission range 410–470 nm) was used for all HPLC procedures. A scanning facility was also available in the ultraviolet region, and this was used to confirm purity and identity of peaks during method development.

Amino acids (native and irradiated) or protein hydrolysates were separated by a C18 5 μm spherisorb reverse phase column. Chromatograms were standardised with native and irradiated amino acids. Elution was performed at a flow rate of 0.75 ml/min using an increasing gradient of acetonitrile in 0.067 M phosphate buffer. After 3 minutes equilibration in phosphate buffer containing 1% acetonitrile, the acetonitrile was increased by 0.6% per minute over 15 minutes, reaching a final concentration of 10% acetonitrile. The column was then re-equilibrated with 1% acetonitrile in phosphate buffer for 10 minutes prior to further injection. The eluent was monitored simultaneously by visible fluorescence and absorption spectroscopy (at 240nm). At 240nm, only aromatic and sulphur containing amino acids (which are the most susceptible to OFR attack) have a native absorbance.

Results

Reverse phase amino acid chromatography

Calibration of the spherisorb ODS column was performed for each batch of analytes using a stock solution containing 4 nmol trp, 25 nmol cys, 80 nmol phe, 15 nmol tyr and 150 nmol met. A typical chromatogram is shown in Fig. 1.

In order to characterise the fluorescent products of oxidised amino acids, pure amino acids were denatured in vitro using different oxygen radical generating systems. Each system has a discrete mode of radical generation, produces different concentrations of OFRs and therefore induces a distinct pattern of molecular decomposition. When aerated solutions of amino acids were exposed to steady gamma radiolysis, fluorophores of trp, cys and phe were detected. However, no fluorescent tyr product could be observed under these conditions. The trp-derived products have been designated as 1, 2 and 3; 1 and 3 being

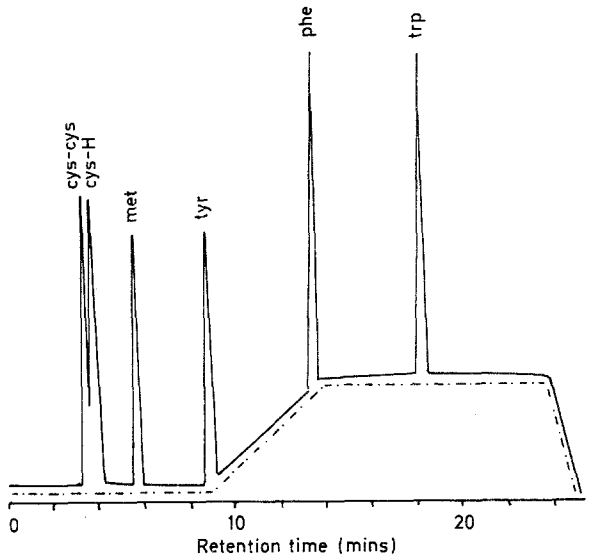

Fig. 1. A schematic representation of a typical chromatogram of 50 ul of native amino acids separated by reverse phase HPLC. UV absorbance at 240 nm is represented by (—) at 0.05 AUFS and visible fluorescence by (—'—) at 0.01 FUFS. The increase in baseline absorbance and fluorescence is due to the acetonitrile gradient in the mobile phase

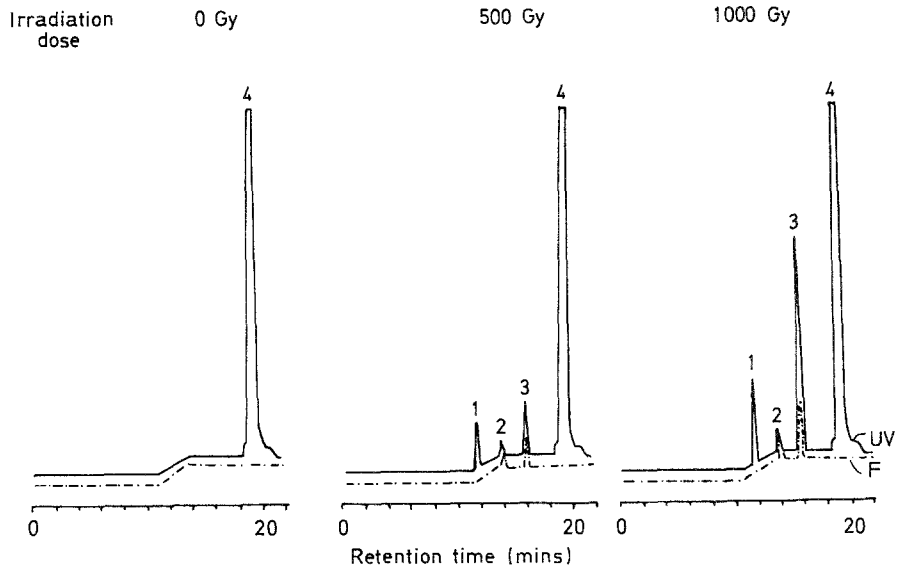

Fig. 2. The dose dependent formation of fluorescent metabolites following gamma radiolysis of tryptophan in aerated solution. Peak identities were confirmed by scanning UV spectroscopy to be; *1* Hexahydropyrolloindole *2* Kynurenine; *3* N-formyl kynurenine; *4* tryptophan

formed in a 2 : 8.6 ratio and Fig. 2 shows a representative chromatogram of trp which has been exposed to radiolytically generated $^{\cdot}OH$ in the presence of oxygen.

Following incubation with H_2O_2 in the presence of CuII, only trp and cys developed any fluorescent products. No fluorophores of oxidised tyr, met or phe

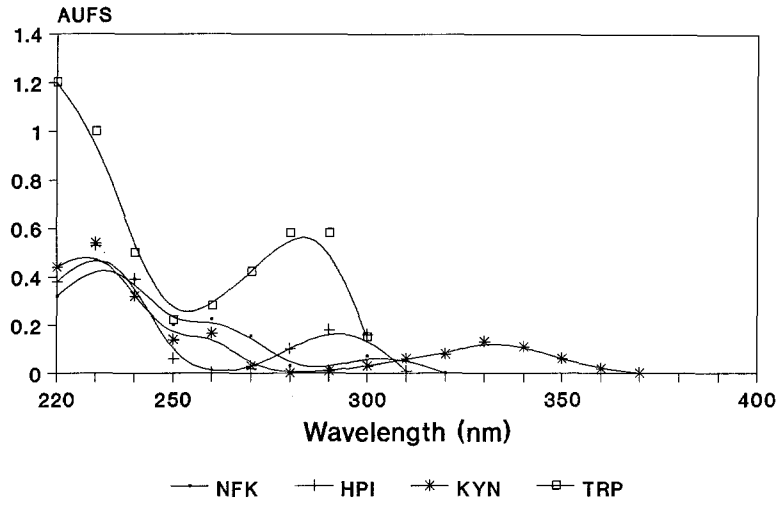

Fig. 3. The absorption spectra of tryptophan and its oxidised metabolites. Scans were taken from 220 nm–370 nm over 15 seconds during routine reverse phase chromatography

were detected under these condition. The photolysis of trp, met, phe and tyr again only yielded fluorescent products derived from trp and cys alone. Under these conditions, the trp products 1 and 3 were formed in a 1 : 2.6 ratio.

Trp was by far the most sensitive amino acid to OFR induced fluorescence formation, and in order to assign identity to the peaks 1, 2 and 3, they were scanned for UV absorbance between 220 and 380 nm. Their absorption spectra are shown in Fig. 3. From a knowledge of their retention times, their relative yields, their characteristic absorption spectra and, where possible, by spiking with authentic standards 1, 2 and 3 have been identified as hexahydro-pyrolloindole (HPI), kynurenine (KYN) and N-formyl kynurenine (NFK) respectively. The limit of detection for the kynurenines by this analytical procedure is 50 pmols. The product formed in greatest amount by all OFR generating systems is NFK and its production is linear with OFR dose (see Fig. 4).

Proteolytic digests of OFR denatured IgG

The denaturation of IgG is believed to be of pathological importance in rheumatoid arthritis (RA) [8]. This, together with its high molar content of trp and tyr, made it a good model protein for studying the oxidative modification of proteins. Separation of the native IgG hydrolysate by reverse phase chromatography yielded 5 major UV absorbing peaks, in the absence of any concomitant fluorescence (see Fig. 5). However, after exposure to $^{\bullet}OH$ in the presence of O_2, a dose dependent increase in the fluorescent products NFK and KYN was observed (see Fig. 6); the latter products being formed in the ratio 8 : 1. $O_2^{\bullet-}$ alone had no detectable effect on amino acids using this analytical procedure. After photolysis at 366 and 254 nm, HPI and NFK were detectable in proteolytic digests of IgG in the ratio of 1 : 10. A major unidentified fluorophore eluted after phe with a retention time of 13.1 minutes, and fluorescence was also generated within phe itself. Site specific $^{\bullet}OH$ generation by H_2O_2 in the presence of Cu again produced

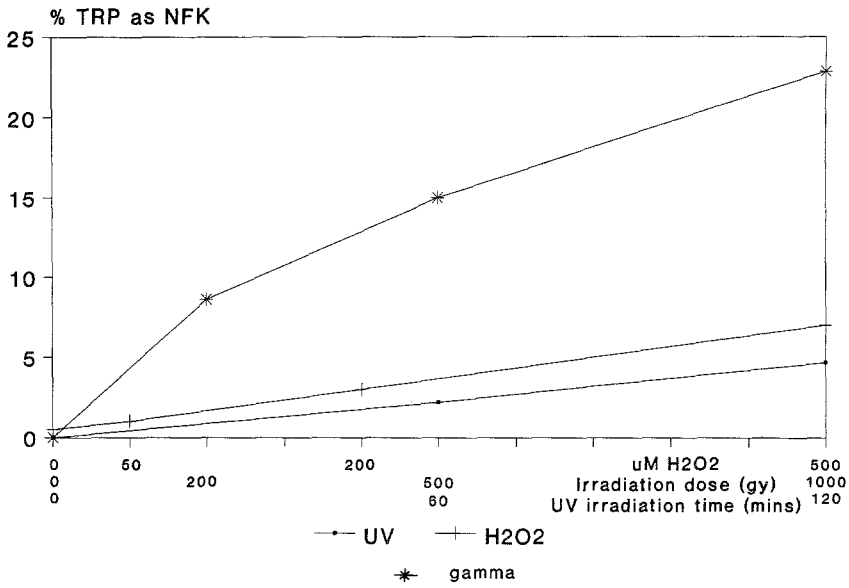

Fig. 4. The generation of N-formyl kynurenine from tryptophan by different sources of OFR showing a dose dependent increase in NFK formation. The between batch coefficient of variation for this procedure was 4.5%

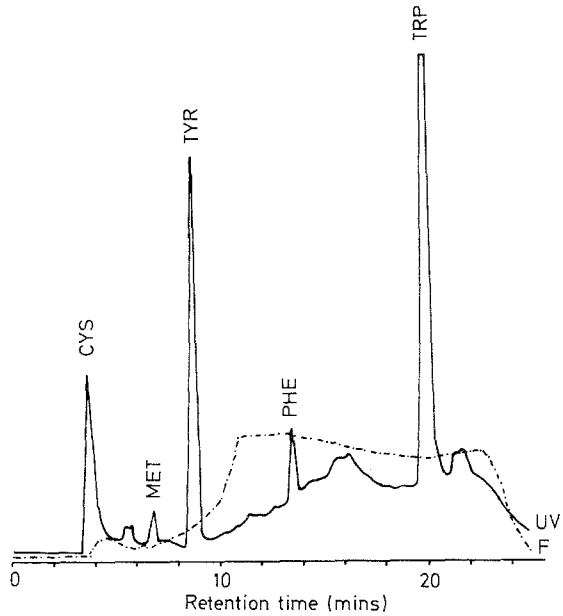

Fig. 5. A typical chromatogram of pronase hydrolysed native IgG (50 ul) as analysed by reverse phase HPLC, showing the five major UV (240 nm) absorbing amino acids. Pronase alone did not yield any amino acid peaks at the concentration and detector sensitivies used

an individual spectrum of oxidised products; NFK and KYN being formed in a 3 : 2 ratio. Table 1 summarises the relative yields of HPI, NFK and KYN produced in IgG by each OFR generating system. As described above for free

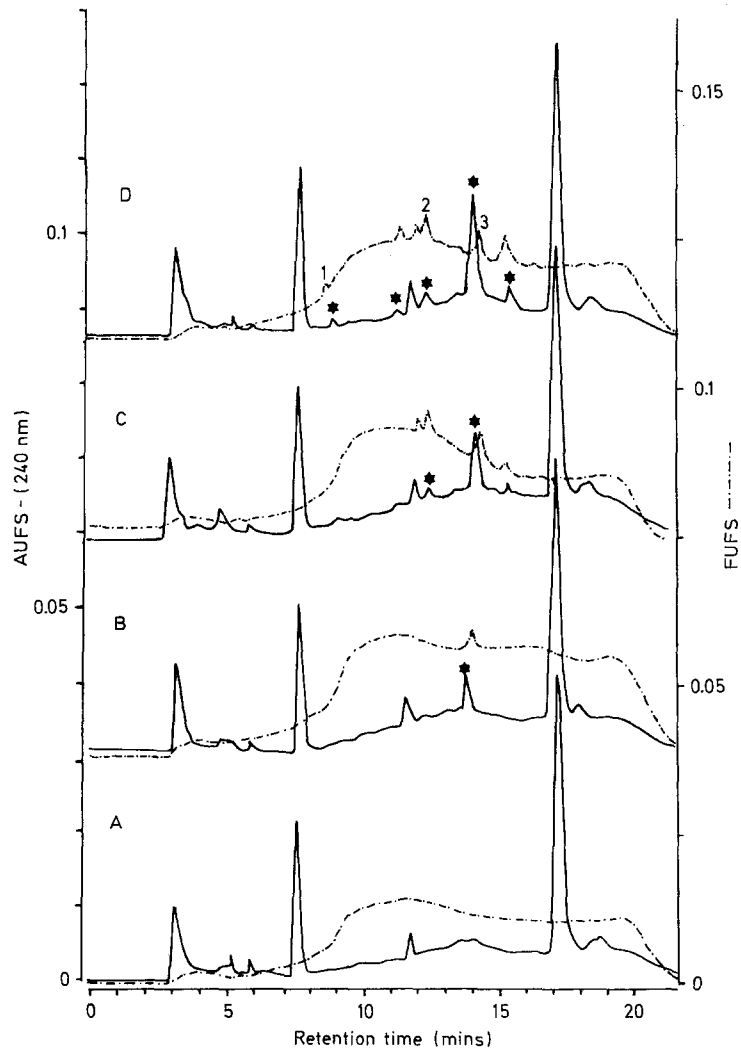

Fig. 6. The effect of increasing dose of radiolytically generated 'OH on the amino acid composition of IgG; (**A**) native IgG, (**B**) IgG + 250 gy, (**C**) IgG + 500 gy and (**D**) IgG + 1000 gy. The appearance of new fluorescent products are represented by *, and three major tryptophan metabolites are identified as 1-HPI, 2-KYN and 3-NFK

Table 1. The ratio of tryptophan metabolite production in IgG following exposure to OFR from different sources, where *UV* represents exposure to 120 minutes of UV irradiation, *Gamma* represents aerated radiolysis of IgG for a dose of 1000 gy and H_2O_2 represents treatment with 5 uM CuII and 500 uM H_2O_2

	HPI	NFK	KYN
UV	1	10	4
Gamma	1	8	1
H_2O_2	2.4	3	2

trp, the generation of NFK within IgG is the most sensitive measure of OFR damage, and its production is again dependent on dose.

Oxidised tryptophan metabolites in OFR treated proteins

In order to study the versatility of the technique in the measurement of general protein oxidation in vivo, the radiolytic decomposition of pepsin and γ lens crystallins was examined. Each protein appeared to have different level of endogenous NFK. However, the exposure of these proteins to $^{\cdot}OH$ in the presence of O_2 generated NFK in a dose dependent manner in all cases as shown in Fig. 7.

The presence of N-formyl kynurenine in serum IgG

IgG molecules purified from the sera of elderly non-rheumatoid subjects, do contain NFK in their protein backbone, however, significantly higher levels of this metabolite were found in rheumatoid serum IgG (see Table 2). Furthermore, NFK concentrations were found to be significantly elevated in synovial fluid IgG when compared to the paired serum IgG.

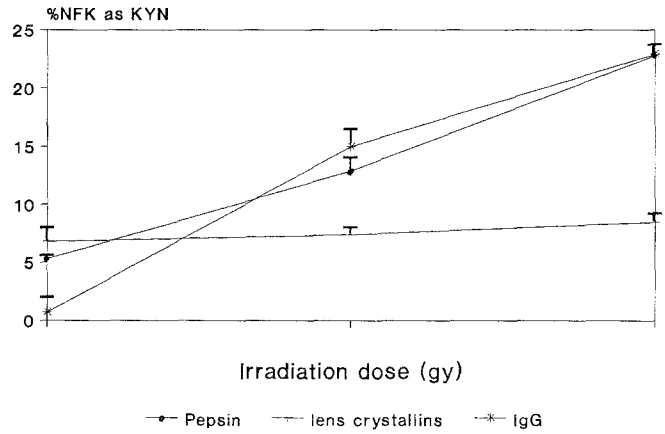

Fig. 7. Dose dependent formation of NFK in OFR treated proteins, showing different endogenous NFK content and its dose dependent increase following simultaneous exposure to $^{\cdot}OH$ and O_2^-. The coefficient of variation for this assay is 4.6%

Table 2. The percentage of tryptophan as NFK in IgG isolated from biofluids. Statistical analysis was by the students "t" test against the elderly control serum, where * represents significance at the 2% level and $^{**}p < 0.01$

Patient group	Mean % Trp as NFK + SD
Elderly controls	5.6 + 0.74
Rheumatoid sera	8.2 + 0.94*
Rheumatoid SFs	10.7 + 0.81**

Discussion

The post-synthetic inactivation of α-1-antitrypsin in acute respiratory distress syndrome has been shown to be a consequence of the loss of one essential methionine residue [30]. These and other studies on glutamine synthetase have demonstrated that OFR target towards specific amino acids on proteins [31], however, such simple amino acids analysis of isolated proteins cannot distinguish whether a phenotypic oxidative modification step has occurred in vivo to the protein as opposed to a pre-synthetic genotypic modification. This is best illustrated by immunoglobulin molecules, since by their very nature as antibodies, they have variable amino acid sequences.

In this paper, we have described a simple HPLC procedure for the analysis of products derived from the oxidation of trp. It does not require the chemical hydrolysis of proteins nor does it require any pre- or post- column derivatisation procedure. Using this technique we have detected the presence of fluorescent products of trp, met and cys following OFR attack. We have specifically identified NFK, KYN and HPI formed after the oxidation of trp in vitro, from their characteristic absorption spectra. We were not able to detect bityrosine nor any other UV absorbing or fluorescent product of tyrosine alone after exposure to any of the OFR generating systems. However, all systems were aerated and this is consistent with the repair of the phenoxyl radical by molecular O_2 [24]: it has been demonstrated that O_2 can scavenge an electron from this intermediate producing $O_2^{\bullet -}$ and regenerating tyrosine, thereby preventing the simple addition of two radical species to produce bityrosine. From these results it seems unlikely that the formation of a bityrosine molecule would occur in vivo, unless, as proposed by Sahlin et al [32] the radical intermediate can be stabilised by an integral haem group. This has been shown to be the case for H_2O_2 mediated crosslinking of sperm whale myoglobin, where the tyrosine radicals required for dimerization are generated by intra-chain electron transfer to the ferryl haem, followed by slow electron transfer from a second tyrosine residue on the adjacent strand [33].

Further evidence disputing the importance of tyrosine oxidation conferring the major fluorescence on an oxidised protein, comes from simple fluorimetric analysis; when equimolar concentrations of trp and tyr were exposed to the same dose of irradiation at physiological pH, the trp fluorescence emission at 430 nm following excitation at 330 nm, was 50 fold greater that of tyrosine (data not shown).

The generation of the fluorophore NFK from trp oxidation was found to be the most sensitive parameter of OFR attack, and, with the exception of $O_2^{\bullet -}$, its rate of formation increased linearly with increasing OFR dose. Thus, NFK appeared to be a potentially useful index of OFR attack, and could be a marker of generalised protein oxidation. This was confirmed by studying OFR denaturation of IgG; in all systems (except radiolytically generated $O_2^{\bullet -}$) dose dependent production of NFK was observed. However, when the ratio of the products HPI : NFK was compared between oxidised trp and oxidised IgG, there was always a lower than predicted yield of HPI in the digested protein. This may reflect the interaction of HPI with other amino acid residues in close proximity,

thereby mediating either inter- or intra-chain crosslinking. Similar suggestions have been made for the interaction of the carbonyls on kynurenines [34] and the amino groups of lysines [35]. The presence of novel crosslinks in IgG is supported by the observation of several novel fluorescent peaks in hydrolysates of OFR treated proteins which could not be ascribed to the oxidation of any one single amino acid. Bityrosine formation has been invoked by many authors to explain the formation of aggregates by OFR attack [25, 14]. However, another possible explanation is the crosslinking of NFK and Lys, or as suggested above, between HPI and another amino acid.

The apparent importance of NFK in conferring visible fluorescence on oxidised IgG may be due to its high molar trp concentration, therefore pepsin (with 6 trp residue/molecule) and γ lens crystallins were studied. Again, when these proteins were exposed to steady state radiolysis in the presence of oxygen, all showed an increase in NFK content with increasing OFR dose.

These findings have confirmed that NFK is formed within a protein backbone following oxidation in vitro, and suggest that this parameter may be a useful index of free radical processes ongoing in vivo. By virtue of the large body of literature describing the possible involvement of OFR in RA, we examined serum and synovial fluid IgG for the presence of NFK. When compared to elderly non-rheumatoid subjects, significantly higher levels of NFK were detected in rheumatoid serum IgG, and paired statistical analysis showed that corresponding synovial fluid IgG was significantly further elevated. It remains to be determined whether such a parameter follows the course of disease or whether it reflects the decrease in antioxidant status [36].

Using this analytical procedure, we have identified the presence of specific fluorescent oxidised metabolites of tryptophan in the absence of any fluorescent products of tyrosine. This establishes the importance of performing such chromatography before ascribing the fluorescence to one specific oxidised amino acid, since fluorescence measurement alone may lead to erroneous conclusions about the identity of the fluorophores. This method has been successfully applied to the analysis of isolated serum proteins, and may be useful in ascribing a role to OFR in disease pathogenesis. Kynurenines have been detected at raised levels in circulating rheumatoid IgG and this work has produced one of the most plausible pieces of evidence for increased OFR generation in vivo and unequivocally linking elevated OFR activity to RA.

References

1. Halliwell B, Gutteridge JMC (1984) Biochem J 219: 1–4
2. Weiss SJ (1986) Acta Physiol Scand [Suppl] 548: 9–37
3. Fantone JL, Ward PA (1982) A J Pathol 107: 397–418
4. Halliwell B, Gutteridge JMC, Blake, DR (1985) Phil Trans R Soc Lond M B311: 659–671
5. Tate RM, Repine JE (1984) In: Pryor WA (ed) Free radicals in biology, vol 6. Academic Press, Florida, pp 199–207
6. Garner MM, Spector A (1980) Proc Natl Acad Sci USA 77: 1274–1277
7. Griffiths HR, Lunec J, Gee CA, Willson RL (1988) FEBS Lett 230: 155–158
8. Lunec J, Blake DR, McCleary SJ, Brailsford S, Bacon PA (1985) J Clin Invest 76: 2084–2090

9. Carp H, Janoff A (1979) J Clin Invest 63: 793–797
10. Wolff SP, Garner A, Dean RT (1986) Trends Biochem Sci 11: 27–31
11. Pryor WA (1986) Ann Rev Physiol 48: 657–667
12. Frankel EN (1980) Prog Lip Res 19: 1–22
13. Lunec J, Griffiths HR, Blake DR (1987) In: Rice-Evans C (ed) Free radicals, cell damage and disease. Richelieu Press, London, pp 151–167
14. Davies KJA (1987) J Biol Chem 236: 397–400
15. Jones AF, Lunec J (1987) Br J Cancer 55 [Suppl]: 60–65
16. Marx G, Chevion M (1985) Biochem J 236: 397–400
17. Wolff SP, Dean RT (1986) Biochem J 234: 399–403
18. Ahn B, Rhee, SG, Stadtman ER (1987) Analyt Biochem 161: 245–257
19. Lunec J (1980) Biochem Soc Proc 10: 21
20. Aloedees HJ, Bloemendal H (1981) In: Bloemendal H (ed) Molecular and cellular biology of the eye lens. John Wiley, New York
21. Creed D (1984) Photochem Photobiol 39: 577–583
22. Creed D (1984) Photochem Photobiol 39: 537–562
23. Singh A, Singh H, Kremers W, Koroll GW (1981) Bull Europ Physiopath Resp 17: 31–41
24. Prutz WA, Butler J, Land EJ (1983) Int J Radiat Biol 44: 183–196
25. Bodaness RS, Leclair M, Zigler JS (1984) Arch Biochem Biophys 231: 461–469
26. Pirie A (1971) Biochem J 125: 203–208
27. Susho P, Pulcini D, Ragone R, Miele L, Della Pietra G, Colonna G (1988) Arch Biochem Biophys 266: 61–71
28. McKinney MM, Parkinson A (1987) J Immunol Methods 96: 291–298
29. Lowry OH, Rosenborough NJ, Farr AL, Randall KJ (1951) Protein measurement with the protein-phenol reagent. J Biol Chem 193: 265–275
30. Tsan MF, Chen JW (1980) J Clin Invest 65: 1041–1050
31. Levine RL (1983) J Biol Chem 258: 11823–11827
32. Sahlin M, Petersson L, Graslund A, Ehrenberg A, Sjoberg BM, Nelander L (1987) Biochemistry 26: 5541–5548
33. Tew D, Cortiz de Montellano, PR (1988) J Biol Chem 263: 17880–17886
34. Freeman BA, J Crapo JD (1982) Lab Invest 47/5: 412–426
35. Jasin HE (1983) J Immunol 130: 1918–1923
36. Lunec J, Blake DR (1985) Free Radic Res Commun 1: 131–139

Authors' address: H. R. Griffiths, Molecular Toxicity Group, University of Leicester, Glenfield Hospital, Groby Road, Leicester LE3 9QP, England.

Received September 21, 1991