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Hypothesis

A mechanism for accelerated degradation of intracellular proteins after limited damage by free radicals

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I propose that limited free radical attack upon proteins, occurring continuously in cells, creates new N-termini (notably aspartate and glutamate) which render the proteins more susceptible to proteolysis by the ubiquitin conjugation system. I suggest that these reactions are a significant part of the previously described 'N-end' and 'PEST' rules, which indicate amino acid termini or sequences which tend to dictate short protein half-lives. I also argue that the N-end rule may apply to sequestered intracellular sites, such as mitochondria, these also being sites of radical generation.

Free radical; Proteolysis; N-terminus; Ubiquitin

1. INTRODUCTION

Free radical fluxes are an inevitable concomitant of cellular metabolism [1–3], for instance of electron transport in mitochondria [4,5] and of microsomal oxidations. They may be accelerated under certain stresses [1–4]. The primary radicals in biological systems are oxygen-centred radicals, such as superoxide, hydroperoxyl and hydroxyl radicals; but secondary radicals can be produced during the autocatalytic process of lipid peroxidation [2]. Both oxygen-centred [3,6–8] and lipid-derived radicals [3,9,10] can damage proteins. Thus cellular proteins may normally be exposed to, and in some cases damaged by, endogenously produced radicals.

When limited radical damage has been inflicted, proteins display amino acid modifications, peptide bond cleavage, and conformational changes [3,6,8]. Peptide bond rupture is particularly relevant to my present argument, and a mechanism for it was first proposed by Garrison [11]. It involves the production of new carbonyl groups in proteins, but does not predict new amino groups at the sites of peptide bond cleavage, unlike enzymatic hydrolysis. It is agreed that carbonyls are produced [3,6,8,11], but we have shown that during low-dose radical attack (e.g. 1–20 hydroxyl radicals per molecule protein) free amino groups are generated [8,12]. This demonstration depends on denaturing proteins and released fragments before measuring amino groups [8,12], and is not possible otherwise [6,13]. After more extensive radical attack, amino groups decrease [3,8,12,13], presumably reflecting fragmentation of the amino acids themselves.

Recently we have confirmed earlier proposals by ourselves [3] and others [7,14] that proline and histidine are important sites of protein fragmentation (Dean, Wolff and McElligott, unpublished). During such reactions the proline and histidine residues of proteins may become converted, respectively, into N-terminal glutamate and aspar-

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tate, as indicated by radioactive precursor-product relationships [15]. The conversion of proline seems rather slow, and is probably encouraged by low pH [14,15]. After such reactions, the proteins, now comprising several polypeptides, may still remain associated, for example during chromatography in non-denaturing conditions [8].

During radical fluxes within cells, many pre-existent antioxidant defences come into play, to limit the extent of damage [1–3]. These include enzymatic defences against the oxygen-centred radicals and their more stable product hydrogen peroxide, and against the lipid hydroperoxides produced during lipid peroxidation; and antioxidant molecules which are stoichiometrically consumed such as those in the lipid phase (for instance α-tocopherol, β-carotene). So normally radical damage is probably quite slight. This can be exemplified by radical-mediated cytolysis [16] and radiation exposure of cells [17] in which quite often no lipid peroxidation is detectable, indicating that antioxidant mechanisms defend adequately, at least in the case of lipids. Nevertheless, products of radical attack on nucleic acids are consistently found in mammalian urine [18], so it is reasonable to assume that slight radical attack upon all macromolecules is occurring continuously.

We have recently shown that during experimentally accelerated radical fluxes in nucleated mammalian cells, accelerated protein degradation can occur [19]. Complementary data are becoming available for erythrocytes and other cells [6]. This accelerated degradation can be construed as a further line of antioxidant defence, supporting those mentioned above [3,6]. It has been noted that after extensive radical damage proteins are so unfolded that, like proteins denatured by a variety of physical techniques, they become more susceptible to in vitro proteolysis [3,6,8] by several proteinases. The relevant proteinases might be within the same cellular compartment as the damaged protein, or might be lysosomal proteinases (active at low pH) if the substrate protein is carried there [20,21] for degradation. Indeed, we have found that susceptibility even to proteinases with low pH optima increases (Hunt and Dean, unpublished). Entry of damaged proteins into the lysosomal system [20] may be aided by unfolding which also causes exposure of increased hydrophobic surface [3,6], which in turn could lead to increased lysosomal membrane binding and hence interiorisation [21].

It is known, however, that accelerated proteolysis can occur under limited (as judged, for instance, by lack of lipid peroxidation) radical stresses [6]. What mechanisms might cause accelerated proteolysis under such moderate (and often physiological) conditions?

2. HYPOTHESIS: THE INTRODUCTION OF DESTABILISING N-TERMINI DIRECTS RADICAL-FRAGMENTED PROTEINS TO THE UBIQUITIN DEGRADATION SYSTEM

Two 'rules' have recently been proposed as being amongst those which dictate rates of catabolism of proteins in cells. The 'N-end' rule [22] indicates that proteins with certain N-terminal amino acids (which are termed 'destabilising') are rapidly degraded in vivo (half-lives less than 2 h). The destabilising amino acids (with their one-letter abbreviation in parentheses) are aspartic acid (D), glutamic acid (E), phenylalanine (F), isoleucine (I), lysine (K), leucine (L), glutamine (Q), arginine (R) and tyrosine (Y, [22]). Conversely, the following amino-terminals are stabilising: alanine (A), glycine (G), methionine (M), serine (S), threonine (T) and valine (V). This rule is based on observations on the different half-lives of β-galactosidase expressed in bacteria with different N-termini and on a survey of N-terminals of long-lived eukaryotic and prokaryotic intracellular proteins from the literature [22]. Recent evidence has suggested that these N-termini may not always be destabilising in themselves, but sometimes (as in the cases of aspartate and glutamate) because they form good substrates for enzymes which incorporate a single basic amino acid into the N-terminus. These enzymes use amino acid-tRNA as donor [23,24]. Thus, terminal aspartate and glutamate can receive additional arginine or lysine termini, which are then destabilising [22]. The second rule is the PEST rule [25], which indicates that sequences rich in proline (P), E, S, and T are present in short-half-life proteins, but not in those of long half-life.

It is striking that the new N-termini envisaged in radical-fragmented proteins [3,7,14,15], glutamate and aspartate, are amongst the most destabilising in the sense of the N-end rule. Thus, I propose that proteins modified in mammalian cells by limited

radical fluxes and so expressing destabilising N-terminal aspartate and glutamate, undergo conversion into proteins with N-terminal lysine and arginine. The products are then susceptible to accelerated catabolism by the ATP-dependent ubiquitin degradative system [23,26]. The importance of proline in PEST sequences [25] may be due similarly to radical fluxes converting some protein prolines into N-terminal glutamate. This effect may be amplified because PEST sequences are surrounded by positively charged amino acid clusters, which often include histidines [25]: these histidines may be converted into N-terminal aspartates, which in turn destabilise the modified proteins.

The specificity of the ubiquitin system is probably determined by the enzyme E3 which conjugates protein substrate to ubiquitin, and whose own specificity is in keeping with these predictions. Thus, free N-termini facilitate the action of E3, particularly if they contain the destabilising amino acids. Termini bearing aspartate or glutamate, which are especially susceptible to modification by amino acid-tRNA transferases into terminal arginine or lysine [24], therefore also facilitate the action of E3 [26]. Conversely, E3 is not avid in binding blocked N-termini [26]. At low-dose radical attack, protein methionines are oxidised to the sulfoxide, which also facilitates binding of the modified protein to E3 and hence catabolism by the ubiquitin system [26].

Under more extreme radical fluxes, perhaps under pathological circumstances, other mechanisms of accelerated proteolysis, both in the organelle in which the protein functions [3,6] and after transport to lysosomes, may collaborate with this effect [3,20,21]. For instance, proteins with blocked N-termini [3,8,12,13] are hardly degraded via ubiquitin [26].

3. SOME PREDICTIONS OF THE HYPOTHESIS

The immediate prediction is that proteins subjected to limited radical modification require tRNA for efficient degradation, and so their degradation in vitro should be inhibited by tRNA degrading nucleases. They should also become more susceptible to incorporation of basic amino acids at their N-termini, by enzymic transfer from amino acid-tRNA donors [24]. During such limited radical fluxes in cells, the ubiquitin-dependent degradation of the proteins should increase, and hence the ATP-dependent proportion may also [26]. In agreement with this prediction, there is evidence of increased ATP dependence of degradation of some radical-modified proteins in nucleated cells [27].

If such mechanisms are a significant contribution to the N-end rule, then they might be expected to apply to any intracellular site at which radical fluxes are occurring: which is thought to be almost everywhere [1–6,20]. For instance, even in those cellular sites which are topologically external (e.g. the interior of mitochondria), radical fluxes may occur [20,28], and so the N-end rule may be applicable. In agreement with this, ubiquitin is present in some such 'external' sites, for example the external face of the plasma membrane [29]. The N-end rule [22] envisages that such external cellular sites show no such rule, or even a rule which is essentially the converse (in which destabilising residues become stabilising). This may be appropriate for extracellular proteins, but the data on which this idea is based seem to contain no examples of such topologically exterior intracellular proteins. I predict that the N-end rule is obeyed by proteins from some topologically external intracellular sites. This is supported by a survey of the NBRF protein sequence database, in which I found that N-terminal destabilising residues are absent in internal mitochondrial proteins, which show long half-lives [30].

Protein sequences for the mature forms of 14 different mitochondrial proteins are available in the NBRF/PIR database. Many mitochondrial proteins are made in the cytoplasm as precursors which undergo proteolytic cleavage(s) to become the mature intramitochondrial protein [31]. Thus, those sequences in the database which are deduced from nucleic acid sequences (which commence with methionine) are the precursor forms and have to be excluded [with the exception of ornithine transcarbamylase (OTC), for which the mature N-terminus (residue 33) is given in the database]. For each available protein I have considered the amino-terminus of the molecule from the most highly evolved eukaryote to have been studied. The data are listed in table 1. There is one protein (cytochrome *c*, human) whose N-terminus is acetylated, which excludes it from consideration in

Table 1

The N-end rule applies to mitochondrial proteins

Protein (and species of origin)	N-terminus
Proteins with stabilising N-termini	
Cytochrome c_1, haem protein (bovine)	S
Cytochrome c_1, non-haem 11 kDa protein (bovine)	G
Ubiquinone-binding protein (bovine)	A
Cytochrome c_1, non-haem 7 kDa protein (bovine)	V
Cytochrome *c* oxidase, polypeptide Va (Baker's yeast)	A
Cytochrome *c* oxidase, polypeptide Vb (Baker's yeast)	V
Elongation factor Tu (rabbit)	T
Malate dehydrogenase (pig)	A
Aspartate aminotransferase (human)	S
ATPase, β-chain (bovine)	S
ATPase inhibitor (bovine)	G
Protein with blocked N-terminus	
Cytochrome *c* (human)	acetylated
Proteins with N-termini which have not been assigned to any stability class	
Ornithine transcarbamylase (OTC, human)	N
Coupling factor 6 (bovine)	N

The data are taken from release 10'0 (1986) of the NBRF/PIR protein sequence database by computer searches for keywords involving mitochondria. Note that the N-terminus of OTC in rat is the stabilising residue S

terms of the N-end rule. Of the remaining 13 proteins, 11 (85%) have stabilising N-termini, supporting my prediction that proteins in compartments separated from the cytosol may still respect the N-end rule.

Two of the proteins (OTC and coupling factor 6) have asparagine (N) N-termini. The N-end rule [22] does not assign N to any category, because of lack of data on degradation of β-galactosidase with N as terminal; the authors anticipate that N is destabilising. Nevertheless, OTC, like most mitochondrial proteins [30], has a long half-life [32]. Whereas OTC in man has N as amino terminus, in the rat it has the clearly stabilising residue, S. In view of this I propose that N is also a stabilising residue.

My hypothesis thus provides an explanation for accelerated proteolysis of proteins after limited radical damage and the mechanisms envisaged may collaborate with others to remove larger quantities of protein during extreme radical fluxes. Furthermore, the hypothesis indicates that radical modification may be a significant determinant of the normal intracellular half-lives of proteins, since it can explain components of both the PEST [25] and N-end [22] rules. The N-end rule may be applicable to compartments segregated from the cytoplasm as well as to the cytoplasm itself.

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