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HISTIDINE AND PROLINE ARE IMPORTANT SITES OF FREE RADICAL DAMAGE TO PROTEINS

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Our hypothesis that proline and histidine are major sites of damage during radical attack upon proteins, becoming respectively glutamate and aspartate, was investigated using proteins biosynthetically labelled with radioactive proline or histidine as targets. Free radicals were generated by copper and H_2O_2, or by gamma radiolysis. Protein-bound histidine was substantially converted into aspartate. Much proline was modified during radical attack, but it was not converted into glutamate. We conclude that histidine and proline are important sites of protein attack by radicals; protein cleavage may result from these reactions.

KEY WORDS: Free radicals, proteins, histidine, proline.

INTRODUCTION

We and others;[1-3] reviewed[4,5] have shown in detail that free radicals can directly fragment proteins, and modify their amino acids. These reactions also cause changes in protein conformation, which can predispose the proteins to proteolysis *in vitro*,[3] in organelles,[6] and in intact cells.[5-7]

Many sites within protins are known to be modified during radical attack. Thus Garrison[1] proposed a mechanism of peptide bond cleavage applicable at any residue. However, there is much evidence that cleavage is more selective.[2,3,8] Schuessler, on the basis of studies with bovine serum albumin,[2] proposed that proline residues may be a major target of fragmentation. In addition, histidine seems to be crucial in the fragmentation of protein occuring when copper and hydrogen peroxide are exposed to protein.[9] This probably results from the unusual ability of histidine to chelate copper and localise the radical generation, but it is not clear whether chain cleavage takes place at, or merely near, the histidines.

We proposed[4] that proline becomes converted into new N-terminal glutamic acid via an oxo-derivative. Similarly,[9] Creeth proposed that histidine may be converted into new N-terminal asparate. Both these reactions could lead to chain breakage. Our purpose here was to study the possible amino acid interconversions directly, by demonstrating precursor-product relationships between radioactive amino acids (histidine and proline) incorporated biosynthetically into proteins, and their products after radical attack upon the proteins *in vitro*.

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MATERIALS AND METHODS

Primary cultures of chick embryo myotubes[10] were labelled in Dulbecco's Modified Eagle's Medium (DMEM) for 48 hours with 1 mCi/ml of either L-[^{14}C(U)]-histidine (347 mCi/mmol) or L-[^{14}C(U)]-proline (266 mCi/mmol), from New England Nuclear. Cells were washed four times with DMEM/1 mM unlabelled histidine or proline, once with Hank's Balanced Salts solution (HBSS) and scraped off into sterile distilled water. Homogenates (1 mg/ml protein prepared by sonication) had less than 4% of the radioactivity soluble in 5% trichloroacetic acid (TCA) and were stored at − 70°C.

The protein mixtures (1 mg/ml; 5 ml) were exposed in 10 mM potassium phosphate buffer, pH 7.2 to radicals generated either with copper (50 μM) and H_2O_2 (5 mM) at 37°C; or by gamma radiation in air (giving a 1:1 ratio of hydroxyl radicals and superoxide radicals[3]) from a ^{137}Cs source (20 Gy/min; 1200 Gy dose). Residual H_2O_2 was removed after the reactions by incubating with 10 mg/ml catalase (Sigma-C-100) at 37°C for 15 min. The generation of TCA-soluble radioactivity during radical attack was less than 20% after radiolysis, and less than 12% after Cu/H_2O_2 attack for 48 h.

A conventional two-stage enzymic hydrolysis at 37°C was used to generate amino acid mixtures from the labelled proteins so as to minimise interconversion of amino acids during hydrolysis. Pronase E (10 μg/ml; Sigma Protease type XXV) was allowed to attack at pH 5 for 24 h; and then leucine amino peptidase (20 μg/ml; Sigma type III-CP) together with prolidase (50 μg/ml), and their cofactors $MgCl_2$ and glutathione (each 1 mM) were added for a further 24 h at pH 7. Hydrolysis was completed by this protocol as judged by: 1) TCA-soluble radioactivity (> 92%; not increased by further hydrolysis); 2) amino group reactivity with o-phthalaldehyde (not increased after 48 h); 3) the failure of fresh proteinases to increase 1) or 2) when added after 48 h; 4) the failure of acid hydrolysis (6N–HCl, 110°C, 24 h) to increase 1) or 2) when performed on the product of the 48 h protocol.

The amino acids present in the radical-modified and control protein samples were analysed specifically for the presence of asp, glu, his and pro, using precolumn derivatisation with o-phthalaldehyde (oPT;[11]). The hydrolysates were precipitated with TCA using 50 μl/ml of 10% w/v bovine serum albumin as carrier. 50 μl of each resultant TCA-supernatant was adjusted to pH 10–10.5 with 4N–NAOH. 30 μl of sodium borate buffer (1 M; pH 10.5) and 20 μl of a solution containing 50 mg oPT, 50 μl of mercaptoethanol and 1 ml of ethanol in 99 ml of borate buffer was added. Normally 2 μl of a 20 mM amino acid standard was also added, thus ensuring a constant ratio of amino groups to aldehyde during reaction. Glutamate was the standard for pro-labelled material and aspartate for his-labelled. The reaction was for exactly 3 min[11] at room temperature, and then 6N–HCl (2 μl) was added to neutralise. The samples (5–10 thousand dpm in 100 μl) were immediately injected into the flowstream of a Waters HPLC apparatus with a C18 μBondapak column (25 × 0.5 cm) equilibrated with 25 mM-ammonium phosphate buffer pH 3.2 (chosen to ensure protonation of the glutamate and aspartate carboxyl groups). Thus the column was run at much lower pH than most authors use (e.g.[11]) so that asp and glu were greatly retarded and eluted after his, in the sequence asp, glu (as shown previously[12]). The retardation of asp and glu increases dramatically and unlike any other amino acids with increasing protonation,[13] and was thus used to confirm the identity of these amino acids: at pH 5.7–7 both asp and glu elute well before his.[11]

The column was run at 1 ml/min, with increasing concentrations of acetonitrile, and compounds of interest eluted during isocratic steps, appropriate for fraction collect-

ing and scintillation counting. Amino acids in the eluent were identified by co-chromatography with authentic standards (Sigma). The elutions were monitored by UV-spectrophotometry, fluorimetry of the oPT derivatives (ex. 340 nm, em. 455 nm) and scintillation counting of fractions. All results shown are from single experiments representative of several.

RESULTS

HPLC Analysis of Standard Proline, Glutamate, Histidine and Aspartate

The elution profile of proline was detected by the radioactive label, since proline (lacking a primary amino group) does not give a fluorescent product with oPT. More than 99.99% of the radioactivity eluted unretarded, thus facilitating testing our proposal that proline becomes converted into an N-terminal primary amino acid, which would be derivatised and hence retarded. We also confirmed that radioactive proline co-eluted with proline standard after derivatisation with 4-chloro-7-nitrobenzofuran (NBD:[14]). Asp and glu gave a single retarded fluorescent peak after derivatisation with oPT: eluting after his under our routine conditions, and before it with conventional elution at pH 5.7 or above.

Radioactive histidine gave a single major fluorescent peak (2 in Figure 1) and other minor fluorescent peaks (including (3) which was also radioactive), as noted in

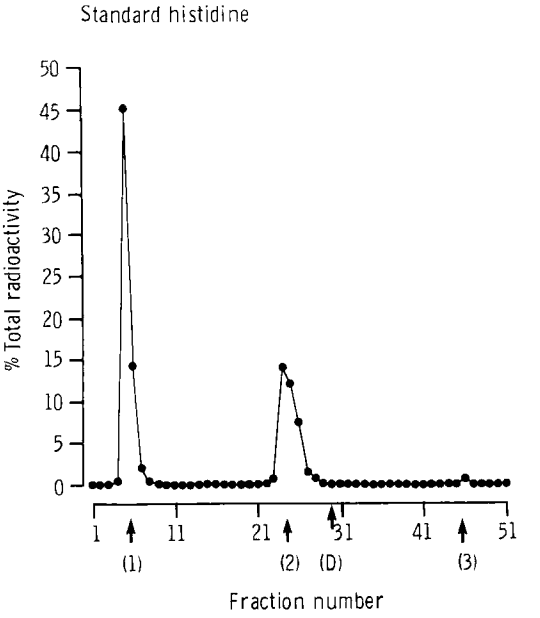

FIGURE 1 ^{14}C-histidine in the presence of unlabelled aspartate was reacted with oPT and analysed by HPLC at pH 3.2 (see Methods). The elution position of aspartate (D) is shown by an arrow. The programmed elution was: 0–12 min, buffer (A); 12–14 min, linear gradient to 25% acetonitrile (B) in A; 14–34 min, 25% B in A; 34–36 min, linear gradient to 50% B in A; 36–52 min, 50% B in A. Fractions were collected once every minute.

previous publicaions (e.g.[11,12]). However most radioactive histidine eluted unretarded and non-fluorescent (peak 1). We performed derivatisation with various concentrations of oPT and histidine: unless histidine was present only at tracer concentrations, peaks 1, 2, 3 were observed in variable proportions, reproducible for a fixed concentration of reactants. None of the histidine peaks coincided with aspartate and thus the method was suitable for detecting the generation of radioactive aspartate from histidine in protein.

Amino Acids in Hydrolysates of Labelled Cellular Protein

When the myotubes were labelled with ^{14}C-proline, the resulting labelled protein hydrolysate contained native proline (unretarded), but two other retarded peaks were also present, probably representing modified prolines present in collagen and other proteins synthesised by these cells.[15] The unretarded peak was confirmed to be proline by derivatisation with NBD.[14] That little radioactive proline was metabolised to other amino acids which became protein-bound is consistent with previous data.[16] The hydrolysate of histidine-labelled cells contained only those radioactive components present in standard histidine. The UV and fluorescent traces of our peaks were extremely sharp; the slight irregularities of peak shapes in the Figures are due to the mixing into relatively large volumes for scintillation counting (as seen in other publications: e.g.[17,18]). The analytical technique established was therefore suitable for the intended purpose of detecting possible conversion of protein-bound histidine and proline to aspartate and glutamate respectively.

Histidine and Proline are Significant Targets for Free Radical Attack on Proteins

Protein-bound histidine was converted to protein-bound aspartate during exposure of the cellular proteins to Cu/H_2O_2 (Figure 2). The conversion measured (by graphical curve stripping on the basis of the aspartate peak) in these conditions with five different preparations of cellular proteins ranged from 5–25%. Conversion was measured with individual protein samples under a range of conditions (Table 1). Conversion required pHs at or above 6.5 (the pK of histidine, below which it chelates copper less well). Once formed, aspartate in protein could be further degraded (Table 1b). This is consistent with out previous demonstration[3] that a net increment in amino groups is generated during low-dose radical attack, but they can suffer a net loss at higher doses (by deamination etc). There were changes in peaks 2 and 3 (Figure 2) in agreement with this, but these were not investigated in detail. Controls lacking Cu/H_2O_2 showed no detectable conversion (i.e. $< 1\%$). During radiolytic radical generation, approx. 2.5% of radioactive histidine in protein was converted to aspartate, and none in parallel non-irradiated controls. In many cases, the identity of aspartate was confirmed by exploiting its idiosyncratic behaviour on HPLC: in elutions in 25 mM-ammonium phosphate at pH 2.5 it eluted even later than shown in Figure 2; and at pH 5.7 it eluted before histidine, as in conventional separations. No aspartate was formed by irradiating free histidine.

When cellular proteins containing radioactive proline were exposed to the Cu/H_2O_2-generated radicals, there was a loss of around 33% of the unretarded peak, with a corresponding increase in the two retarded peaks. During radiolytic attack 25% of the unretarded proline was converted into the two retarded moieties. The elution profiles of both the control and the radical-stressed hydrolysates were unaffected by

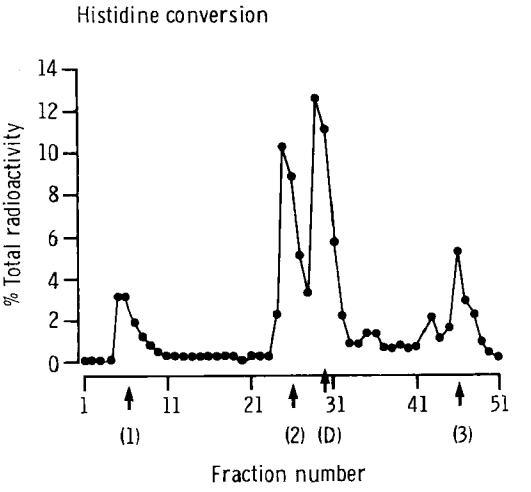

FIGURE 2 A ^{14}C-histidine-labelled myotube homogenate was exposed to 50 μM-$CuSO_4$ and 5 mM-H_2O_2 for 24 h at 37°C in distilled water titrated to pH 6.5. The products were enzymatically hydrolysed, derivatised and analysed by HPLC at pH 3.2 as described in Methods. No histidine conversion (i.e. < 1%) was observed in a corresponding control incubated without copper and peroxide. The elution position of aspartate (D) is shown by an arrow. Fractions were collected once every minute.

TABLE 1
Conversion of protein-bound histidine to aspartate during radical attack on cellular proteins

Conditions	% Conversion
A:	
Low pH (10 mM-sodium acetate buffer, pH 5.0)	< 1
Unbuffered water (pH adjusted to 6.5)	5.5
10 mM-potassium phosphate buffer, pH 7.2	4.5
B:	
10 mM-potassium phosphate buffer, pH 7.2	
Duration of incubation (hours):	
0	< 1
6	7.2
24	4.5
48	4.4

^{14}C-histidine-labelled myotube proteins were subjected to radical attack as defined in Methods. The smallest conversion detectable was 1%, and all the control incubations (lacking copper and H_2O_2) failed to reach this limit.

A: Radical attack for 24 h at 37°C; 50 μM-$CuSO_4$ and 5 mM-H_2O_2 were added.

B: Radical attack in 10 mM phosphate buffer pH 7.2, for the various indicated times at 37°C, in the presence of the same radical-generating components.

the derivatisation with oPT: thus none of the peaks corresponded to molecules containing primary amino groups. It was confirmed that neither retarded moiety coincided with glutamine. Thus protein-bound proline was also a major target of radical attack; peak 2 may correspond to the oxo-derivative we postulated[4] but none of the product coeluted with glutamate. Acid hydrolysis of the radical-modified proteins, in contrast, did generate detectable glutamate, suggesting that the inter-

mediates produced by radical attack are labile under extremely acid conditions, and give rise then to some glutamate.

DISCUSSION

In previous preliminary work;[19] and (unpublished data of SPW, RTD and D. Renouf) we found that hydroxyl radical attack (in the presence of oxygen) on bovine serum albumin and collagen (type 1, acid soluble from calf skin) leads to losses of proline and histidine, and increases in aspartate and glutamate (as judged in conventional amino acid analyses after acid hydrolysis). Other workers have recently reported data on albumin[8,20] and apoprotein B of low density lipoprotein[21] in agreement with this. In addition, aspartate has been detected in preliminary studies of amino acids derived from labelled histidine within glutamine synthetase by limited mixed-function oxidation.[18] However, these data all have the disadvantage of using acid hydrolysis (which modifies some amino acids, and may have a range of actions on the amino acid derivatives produced during radical attack), and they cannot[8,20,21] or do not attempt[18] to demonstrate precursor-product relationships. In contrast, the experiments reported here show directly that radical attack converts histidine residues in proteins to aspartate, providing they are unprotonated (pH 6.5 or above). This reaction may result in protein fragmentation, in agreement with other recent data.[22] The aspartate could be further degraded, as noted in previous studies.[3–5] Proline was also a major target of radical attack, but was not converted into glutamate. Chain cleavage probably results from these reactions of proline,[4] especially at low pH, for instance, in lysosomes.[23]

The residues involved and produced in these reactions of free radicals with proteins are probably important determinants of degradation rates of proteins. Histidine[18] and proline[24] may be determinants of degradation rates of *intact* proteins; while aspartate and glutamate may be important in determining the fate of radical-modified proteins.[23] Since our data also demonstrate the generality of the reactions studied, it should be possible in future work to use the assay of production of some of the derivatives of histidine and proline as criteria for the extent of radical damage to proteins in living cells. Many other sites of reactions of free radicals with proteins may also result in fragmentation of the proteins[1] but these probably have lesser bearing on the subsequent fate of the proteins.

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