THE IDENTIFICATION OF TRYPTOPHAN RESIDUES IN PROTEINS AS OXIDISED DERIVATIVES DURING AMINO ACID SEQUENCE DETERMINATIONS

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1. Introduction

Although tryptophan residues in peptides and proteins have been successfully identified using the automatic Edman procedure [1], it has been observed that tryptophan becomes more difficult to identify as the number of Edman cycles increases [2]. Tryptophan is completely destroyed during the hydrolysis step of the manual dansyl procedure [3] and the use of other hydrolytic reagents [4,5] has been recommended to preserve this labile amino acid residue. During photochemical and chemical treatments of peptides and proteins [6–8], tryptophan forms oxidation products that are moderately stable; kynurenine for example, is stable to 6 N HCl at 108°C under vacuum [9]. It seemed therefore that it should be possible to prepare phenylthiohydantoin (PTH) and dansyl derivatives of some tryptophan oxidation products. If so, these derivatives could be used either to identify oxidation products of tryptophan after chemical or photooxidation reactions, or to identify tryptophan in peptides and proteins using amino acid sequencing techniques.

The results of our experiments show that it is possible to derivatize the common tryptophan oxidation products, and suggest that initial oxidation of tryptophan residues to either oxindolyl-3-alanine with dimethylsulfoxide/HCl [7] or kynurenine with sodium periodate [10] would facilitate the identification of tryptophan when using the Edman degradation procedure. Oxidation to oxindolyl-3-alanine is also the first step of a two-step tryptophan cleavage procedure [11] and hence portion of the oxidised protein could be used for subsequent cleavage by this specific fragmentation method. Kynurenine forms a dansyl derivative which is stable to HCl hydrolysis. PTH-kynurenine is readily identified by its fluorescence at 360 nm, or after hydrolysis to the free amino acid with HI [12].

2. Materials and methods

Four common tryptophan oxidation products (see fig.1), kynurenine (Kyn), oxindolyl-3-alanine (Oia), dioxindolyl-3-alanine (Dia) and 3a-hydroxy-1,2,3,3a, 8,8a-hexahydropyrrolo (2,3,6)-indole-2-carboxylic acid (Hpi), were synthesized [8,13] and successfully converted to the respective phenylthiohydantoins

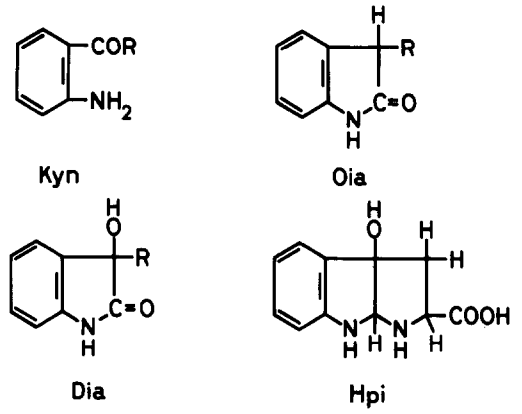

Fig.1. Tryptophan derivatives synthesized for the sequence studies. R = $CH_2CH(NH_2)COOH$, Kyn = kynurenine, Oia = oxindolyl-3-alanine, Dia = dioxindolyl-3-alanine, Hpi = 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo(2,3,6)-indole-2-carboxylic acid.

[1]. The peptide Ala–Gly–Trp–Leu [6] was converted to Ala–Gly–Oia–Leu by treating with a mixture of dimethylsulfoxide and 12 M HCl at room temperature for 15 min [7], purification being achieved by chromatography on Sephadex LH 20 with water as eluent. Apomyoglobin was converted to Oia-apomyoglobin similarly. The peptides Gly–Kyn–Gly and Kyn–Gly were synthesized [13], Ala–Gly–Kyn–Leu was prepared by oxidation of Ala–Gly–Trp–Leu with 10 molar equivalents of sodium periodate for 16 hr at room temperature and purified by ion exchange chromatography. Kyn-apomyoglobin was prepared similarly by oxidising apomyoglobin, purifying by dialysis (cf. [10]). (This procedure would actually convert tryptophan to formylkynurenine but the formyl group is labile under acid conditions.) This procedure was considered to be more convenient than alternative methods such as ozonolysis [14] or photooxidation [15].

Automatic sequence determinations were made as described previously [16,17]. Manual sequencing was carried out using the method of Hartley [3], except that hydrolysis of the dansyl derivatives was carried out at 108°C for only 6 h.

3. Results and discussion

The four PTH-derivatives prepared from tryptophan oxidation products were distinguishable from the PTHs of the common amino acids by thin-layer chromatography (see fig.2) [18]. PTH-kynurenine, which ran just ahead of PTH-methionine, exhibited a strong fluorescence under long wavelength UV light (360 nm). PTH-oxindolyl-3-alanine was well separated from other PTH-derivatives, appearing between PTH-tyrosine and PTH-(N_ϵ-PTC)lysine. PTH-dioxindolyl-3-alanine gave two spots (presumably due to the presence of diastereoisomers [8]), one before and one after PTH-aspartic acid. PTH-Hpi chromatographed just ahead of PTH-tryptophan. All the PTH-derivatives gave strong purple colours when sprayed and heated at 100°C with ninhydrin-collidine followed by copper nitrate [18].

Amino acid sequence determinations using the automatic sequenator and thin-layer chromatography established that all peptides were degraded normally and only PTHs corresponding to the amino acids in the peptide were found at each cycle. For Oia-apomyoglobin, in addition to the expected PTH-oxindolyl-3-alanine at residues 7 and 14 there was evidence for a small amount of tryptophan (less than 10% as estimated from a 270 nm scan of the thin-layer chromatography plate [19]), indicating that the oxidative treatment was not quantitative under the conditions used. Amino acid analysis showed that conversion was virtually quantitative for Kyn-apomyoglobin and fig.3 illustrates the easy identification of

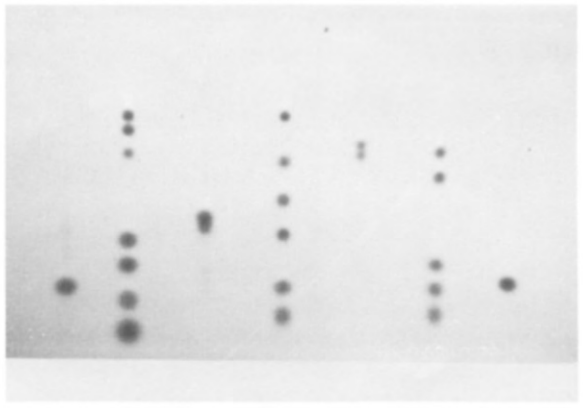

Fig.2. Photographs under UV light (254 nm) of a thin-layer chromatography plate after chromatography of PTH-Kyn, PTH-Oia, PTH-Dia and PTH-Hpi with PTH reference mixtures [13] for comparison.

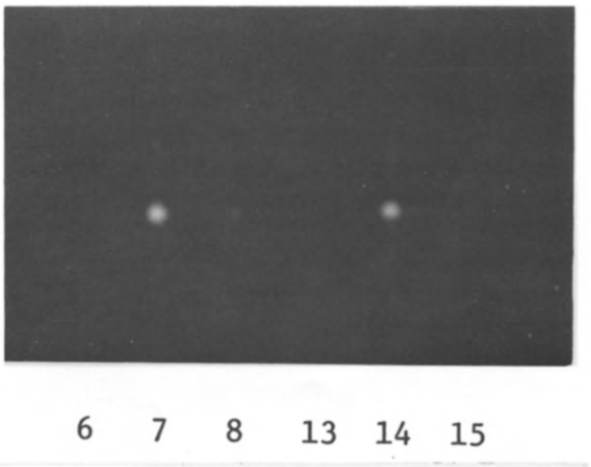

Fig.3. Photograph under UV light (360 nm) of a thin-layer chromatography plate after chromatography of residues 6,7,8,13,14,15 of kyn-apomyoglobin from the protein sequenator.

PTH-kynurenine at residues 7 and 14 under 360 nm light. With these residues, however, there was a small amount of a pinkish-coloured substance on the plate with an R_F similar to PTH-leucine. These results therefore confirm that the Edman degradation procedure provides a means of establishing the effect on tryptophyl residues of various oxidative treatments of peptides and proteins.

Conversion of tryptophan to either kynurenine or oxindolyl-3-alanine should produce an amino acid residue that is more stable to repetitive Edman degradations. Oxidation of tryptophan to oxindolyl-3-alanine with dimethylsulfoxide/HCl is the more specific treatment, the only other amino acid affected being methionine which is oxidised to methionine sulfoxide [7].

Oxidation of tryptophan to kynurenine using periodate results in oxidation of cystine and partial oxidation of histidine, tyrosine and N-terminal serine and threonine [20]. Amino acid analysis after perodate oxidation of apomyoglobin indicated that there was a 10% loss of histidine and a 20% loss of tyrosine, although identification of histidine at residue 12 presented no difficulties. PTH-kynurenine has the advantage that it fluoresces at 360 nm and thus is more easily identified by thin-layer chromatography than PTH-oxindolyl-3-alanine. Analysis of the products from the HI hydrolysis of PTH-kynurenine on the amino acid analyzer revealed the presence of two components, the expected peak of kynurenine followed by a larger peak which has been tentatively identified as σ-aminohomophenylalanine. The latter

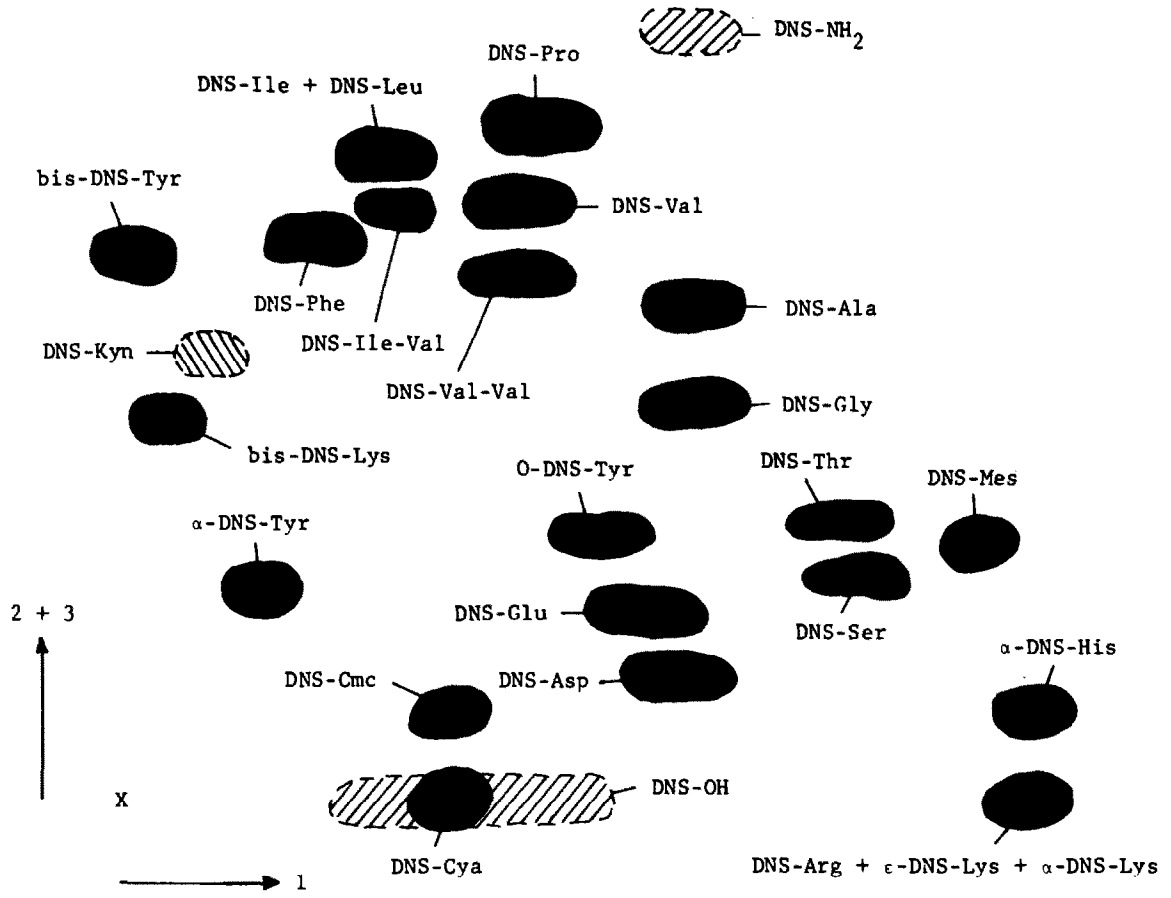

Fig.4. Diagram of position of dansyl-Kyn relative to common dansyl amino acids after chromatography in 3 solvent systems [3].

component presumably arises from reduction of the carbonyl group during HI hydrolysis. The dansyl derivative of kynurenine is stable to HCl hydrolysis and is well resolved from the common dansyl amino acids (see fig.4) [3]. Thus it could be identified using the manual dansyl sequencing procedure in the peptides Kyn–Gly, Gly–Kyn–Gly and Ala–Gly–Trp–Leu after periodate oxidation.

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References

[1] Edman, P. (1970) in: Protein Sequence Determination, (Needleman, S. B. ed) Springer-Verlag, Berlin.
[2] Hunkapiller, M. W. and Hood, L. E. (1978) Biochemistry 17, 2124–2133.
[3] Hartley, B. S. (1970) Biochem. J. 119, 805–822.
[4] Flengsrud, R. (1976) Anal. Biochem. 76, 547–550.
[5] Giglio, J. R. (1977) Anal. Biochem. 82, 262–264.
[6] Holt, L. A., Milligan, B., Rivett, D. E. and Stewart, F. H. C. (1977) Biochim. Biophys. Acta 499, 131–138.
[7] Savige, W. E. and Fontana, A. (1977) Methods Enzymol. 47, 442–453.
[8] Savige, W. E. (1975) Aust. J. Chem. 28, 2275–2287.
[9] Previero, A., Signor, A. and Bezzi, S. (1964) Nature 204, 687–688.
[10] Atassi, M. Z. (1967) Arch. Biochem. Biophys. 120, 56–59.
[11] Savige, W. E. and Fontana, A. (1977) Methods Enzymol. 47, 459–469.
[12] Inglis, A. S., Nicholls, P. W. and Roxburgh, C. M. (1971) Aust. J. Biol. Sci. 24, 1247–1250.
[13] Rivett, D. E. and Stewart, F. H. C. (1976) Aust. J. Chem. 29, 2095–2100.
[14] Previero, A., Previero, C. and Jolles, P. (1966) Biochem. Biophys. Res. Comm. 22, 17–21.
[15] Galiazzo, G., Jori, G. and Scoffone, E. (1968) Biochem. Biophys. Res. Comm. 31, 158–163.
[16] Crewther, W. G. and Inglis, A. S. (1975) Anal. Biochem. 68, 572–585.
[17] Inglis, A. S. and Burley, R. W. (1977) FEBS Lett. 73, 33–37.
[18] Inglis, A. S. and Nicholls, P. W. (1973) J. Chromatogr. 79, 344–346.
[19] Inglis, A. S., Nicholls, P. W. and Strike, P. M. (1975) J. Chromatogr. 107, 73–80.
[20] Clamp, J. R. and Hough, L. (1965) Biochem. J. 94, 17–24.