Analysis of the peptide carrier in the scutellum of barley embryos by photoaffinity labelling

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Abstract. The preparation of a phenylalanine analogue containing an azido group and its incorporation into dipeptides is described. Peptides modified in this way are taken up into barley (Hordeum vulgare L.) scutella via the previously characterized peptide-transport system. Photoactivation of modified peptides in the presence of isolated scutella resulted in irreversible inhibition of peptide uptake in a concentration-dependent manner. Transport of other solutes which share a common mechanism of energy coupling, but which are transported via distinct carriers, was not inhibited after photo-derivatization of scutella with the modified peptides. Derivatization of isolated scutellar tissue with a 14C-labelled peptide analogue, resulted in incorporation of label into two proteins of Mr = 54000 and 41000. Scutellar tissue from early-germinating seeds, which do not show active peptide uptake, did not incorporate label into these polypeptides. It is concluded that these proteins are components of the barley peptide-transport system.

Key words: Embryo (peptide carrier) – Hordeum (embryo, peptide transport) – Peptide transport – Photoaffinity probe

Introduction

Germinating barley seeds and other cereals mobilise their storage proteins by enzymic hydrolysis into a mixture of small peptides and amino acids (Higgins and Payne 1981; Enari and Sopanen 1986; see review by Fincher 1989). These soluble products are subsequently absorbed by the embryo via the scutellum (Higgins and Payne 1980; Walker-Smith and Payne 1984a, 1985; Sopanen et al. 1985; Salmenkallio and Sopanen 1989).

The uptake of peptides appears to be via either a single carrier (Higgins and Payne 1978a) or two overlapping systems both of which show broad specificity (Sopanen 1984). Initial studies showed peptide uptake to be stereospecific, energized by a proton gradient and behaving according to Michaelis-Menten kinetics (Higgins and Payne 1977, 1978b; Sopanen et al. 1978).

Analysis of the protein component(s) of the peptide-uptake system showed that the carrier contained essential paired thiol groups at the substrate-binding site. This property was used to introduce radioactive thiol ligands into the transport proteins, which allowed localisation of the carrier to the plasma membrane of the scutellar epithelial cells (Walker-Smith and Payne 1983, 1984b). A procedure developed to label specifically only vicinal dithiols with p-chloro-2Hgmercuribenzenesulphonic acid (p-CMBS), followed by extraction and analysis of labelled proteins, led to the identification of two polypeptides as possible components of the peptide-uptake system (Payne and Walker-Smith 1987). The use of thiol-directed probes is far from ideal, however, due to the occurrence of these groups on various proteins. This, limitation and the subsequent unavailability of the commercial radioactively labelled p-CMBS, has led us to develop alternative means for labelling to permit identification of the protein(s) involved in peptide uptake.

To be able to label a specific protein in a complex biological tissue requires interaction between a unique site on a protein and the label. The technique of photoaffinity labelling fulfils this requirement, providing a suitable substrate analogue can be prepared. One of the most commonly used active moieties in photolabelling studies is the nitrene radical, which is most commonly derived from photolysis of aryl- or nitro-aryl azides (Bayley and Knowles 1977). Azido derivatives of phenylalanine (Phe) have been used as photoaffinity probes for amino-acid carriers (Noren et al. 1983; Schlichter and Bajorat 1990) and also for dipeptide uptake in Escherichia coli when
combined into the dipeptide, Gly-Phe (Staros and Knowles 1978). In the rat intestine, a membrane glycoprotein of $M_r = 127,000$ has been labelled with photoactivatable derivatives of Gly-Pro and of the $\beta$-lactam antibiotic cephalaxin, which are purported to share a common transport system (Kramer 1987; Kramer et al. 1990a).

This paper reports the preparation and use of peptide analogues containing azido-modified phenylalanine as photoaffinity probes of the peptide-uptake system in germinated and germinating barley embryos.

Materials and methods

[U-$^{14}$C] Alanine (Ala) (6.0 GBq $\cdot$ mmol$^{-1}$) and Gly[U-$^{14}$C]Phe (474 MBq $\cdot$ mmol$^{-1}$) were from Amersham International, Amersham, Bucks., UK. Phenylsine oxide was from Fluka Chemie AG, Buchs, Switzerland. All other laboratory chemicals were from Sigma Chemical Co. or BDH, both of Poole, Dorset, UK, and were of the highest purity available. Al[ U-$^{14}$C]Phe (2.0 GBq $\cdot$ mmol$^{-1}$) was prepared in our laboratory by coupling [U-$^{14}$C]Phe (15.7 GBq $\cdot$ mmol$^{-1}$, Amersham International), with N-t-butoxycarbonyl (Boc)-Ala-$^{N}$-hydroxyxuccinimide ester as described by Staros and Knowles (1978). Aluminium-backed silica gel TLC plates (20 cm $\times$ 20 cm) were from BDH. Nitrocellulose paper (Hybond C) and autoradiography film (Hyperfilm–3H) were from Amersham International. Film developer (LX-24) and fixative (FX-40) were from Kodak Hemel Hemsted, Hefts., UK. Soluene-100 tissue solvent was from Packard Instrument Co. Inc., USA. Barley (Hordeum vulgare L., Maris Otter, Winter) seeds were from the National Seed Development Organisation, Cambridge, UK.

Preparation of 4-azido-2-nitro-phenylalanine. 4-Azido-2-nitro-phenylalanine hydrochloride (Phe($N_3$NO$_2$)·HCI) was synthesized with minor modifications by the method of Staros and Knowles (1978). The product gave a single spot by TLC as described below ($R_f$ of Phe($N_3$NO$_2$)·HCI in System A 0.31–0.34, System B 0.57–0.64) and gave a single peak on reverse-phase chromatography [Pharmacia PEP-RPC (C$_18$)] column equilibrated with aqueous trifluoroacetic acid (TFA) (0.1%) and eluted with a gradient of CH$_3$CN containing TFA (0.1%) and eluted with a gradient of CH$_3$CN (1978). The product gave a single spot by TLC as described below ($R_f$ of Phe($N_3$NO$_2$)·HCI in System A 0.31–0.34, System B 0.57–0.64) and gave a single peak on reverse-phase chromatography [Pharmacia PEP-RPC (C$_18$)] column equilibrated with aqueous trifluoroacetic acid (TFA) (0.1%) and eluted with a gradient of CH$_3$CN containing TFA (0.1%). Elution occurred at 18% CH$_3$CN and was monitored by absorbance at 214 nm. Typical yields were: 5 g of p-amino-phenylalanine gave 2.06 g of 4-amino-2-nitro-phenylalanine (32.8%); 2.06 g of 4-amino-2-nitro-phenylalanine gave 0.305 g of 4-azido-2-nitro-phenylalanine (11.6%). The infrared spectrum of 4-azido-2-nitro-phenylalanine (KBr disc) was strongly absorbing bands at 2130 cm$^{-1}$ (azido-nitro derivative) and 1740 cm$^{-1}$ (hydrochloride salt) (Dolphin and Wick 1977).

Preparation of glycyllalanyl-4-azido-2-nitro-phenylalanine. Gly- and Ala-Phe($N_3$NO$_2$) were synthesised by the method of Staros and Knowles (1978) with minor modifications. The Boc-dipeptides, after partitioning into ethyl acetate as described, were dried down and deblocked by treatment with ice-cold anhydrous TFA for 60 min. The TFA was removed in a stream of nitrogen and the residue redissolved in aqueous TFA (0.1%). Final purification of the free peptide occurred at 18-20% CH$_3$CN concentration. Typical yield was about 45%. Thin-layer chromatography in System A gave $R_f$ 0.64 for Boc-[U-$^{14}$C]Phe and $R_f$ 0.09 fr [U-$^{14}$C]Phe. Purified material was freeze dried for use in the next step. Preparation of the activated ester of Boc-[U-$^{14}$C]Phe and its coupling to Phe($N_3$NO$_2$) was as described by Staros and Knowles (1978) for the glycyll peptide. The Boc-dipeptide was deblocked with cold TFA as above and free peptide purified by reverse-phase chromatography. Elution of the free peptide occurred at 18-20% CH$_3$CN concentration. Typical yield of [U-$^{14}$C]Ala-Phe($N_3$NO$_2$) from Boc-[U-$^{14}$C]Phe was about 50%. The peptide was dissolved in water and stored at $\sim$20 °C in darkness.

Analysis by TLC. Silica-gel-60 plates were developed in either 1-butanol/acetic acid/water (90:10:25, by vol.) (System A) or chloroform/methanol/ammoniaaq (50:50:10, by vol.) (System B). Plates were air dried and sprayed with ninhydrin reagent (0.2% w/v in ethanol) followed by warming to detect spots. Labelled compounds were detected by their activity using a TLC-plate radioscanmer (TLC Scanner II; Berthold/Freske, Wildbad, Germany).

Plant material. Barley seeds were germinated on 1.2% (w/v) aqueous agar at 23 °C in darkness for 5 h or 24–30 h. The scutella were excised with a scalpel into sodium phosphate-citrate buffer (100 mM) pH 3.8 as previously described (Walker-Smith and Payne 1984a).

Solute-uptake assays. Rates of peptide uptake into isolated scutella were measured from a solution of peptide (2 mM) containing 1.85 kBq $\cdot$ ml$^{-1}$ of label as described previously (Walker-Smith and Payne 1984a). In addition, for uptake of the azido-modified peptide analogue only, the level of label remaining in the tissue after extraction with acetic acid was determined. This was achieved by complete solubilization of the tissue in 250 μl of ‘Soluene’ tissue disintegrator, followed by scintillation counting. Uptake of [U-$^{14}$C]Glycine was measured as for peptide uptake but substituting amino acid in the incubation mixture, and uptake of n-[U-$^{14}$C]Glucose was from a solution of glucose (20 mM) containing 8.88 kBq $\cdot$ ml$^{-1}$ of label, measured as for peptide uptake.

Photoaffinity labelling of tissue with peptide analogues

Whole scutella: Isolated scutella were incubated (see text for exact details of each experiment) in a solution of peptide in sodium phosphate-citrate buffer (50 mM, pH 3.8) in a 1-ml quartz cuvette, placed against the tube of a long-wavelength UV light source (Andermann, Kingston-upon-Thames, Surrey, UK) and surrounded with aluminium foil. Air was bubbled through the solution via a hypodermic needle both to aerate and to stir the solution. After a brief incubation in darkness the mixture was photoactivated by exposure to the UV source. Control experiments were performed in the same way but without added peptide. Following photoactivation, scutella were washed thoroughly in water, then stored in buffer (pH 3.8) until assayed for their solute uptake capability (< 30 min).

Isolated epithelial tissue: Photoaffinity labelling with radioactively labelled peptide analogue was also performed on isolated epithelial tissue. In this case, embryos were isolated from germinated grains and the epithelial surface of the scutellum removed by gently scrapping with a scalpel blade. Isolated epithelial tissue was stored in ice-cold sodium phosphate-citrate buffer (pH 3.8) until use (up to 30 min). Photoaffinity labelling was carried out with the tissue suspended in a solution of [U-$^{14}$C]Ala-Phe($N_3$NO$_2$) in buffer (45 mM, pH 3.8) and photoactivated with UV light, as described above.

Membrane preparation and extraction of proteins. Isolated epithelial tissue from 15–40 scutella was homogenised using a glass homoge-