Peptide uptake in germinating barley embryos involves a dithiol-dependent transport protein

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An active transport system for small peptides occurs in the scutellar membrane of germinating barley and serves to move the products of partial hydrolysis of storage proteins from the endosperm into the growing embryo. Transport of peptides, but not amino acids or glucose, is inhibited by the thiol reagents, *N*-ethylmaleimide and *p*-chloromercuribenzene sulphonic acid (PCMBS). Peptide substrates protect against PCMBS inactivation. The dithiol-specific reagent, phenylarsine oxide (PAO) also inhibits. The reducing agent, dithiothreitol, reverses the inactivation caused by PCMBS and PAO. We conclude that the peptide transport system contains a redox-sensitive, dithiol-dependent protein.

Germinating barley *Peptide transport* *Redox-sensitive dithiol* *Sulphydryl-dependent transport*

1. INTRODUCTION

In seeds, nitrogen reserves occur primarily as special storage proteins that are laid down in the endosperm. On germination, enzymes are released from the surrounding aleurone layer into the endosperm to hydrolyse these polymers. The hydrolysis products are then absorbed into the growing embryo, where they are used during development for the synthesis of roots, shoots, and the like. In a monocot seedling such as barley, the endosperm is separated from the embryo by a special absorptive tissue, the scutellum, across which all such nutrient transfer must occur.

Until quite recently it had been assumed that nitrogen reserves were transferred into the embryo in the form of amino acids, resulting from complete hydrolysis of storage proteins (reviews [8,10]). However, appreciation of the inherent advantages of transporting peptides relative to amino acids [12], and noting the general absence of peptidases in the endosperm and their presence in the embryo, the scutellum was examined for the possibility that it might transport peptides also: this was shown to be the case [1,3,13,17]. Since then similar systems have been found in other cereals such as wheat, oats, sorghum and maize (unpublished).

The broad characteristics of peptide uptake by barley scutellum have been established. Transport is active and accumulation is against a concentration gradient [1,3,17], it shows a pH optimum at about pH 4 [4,18], which is the ambient pH of the endosperm and the optimum for endosperm proteolytic activity. Uptake is sensitive to a range of metabolic and respiratory inhibitors [4,18]: acetate, at a pH below its pK, also specifically inhibits uptake, this being compatible with the dependence of the system on maintenance of a proton gradient [4]. No evidence has been found for a Na^+ dependence for uptake [4,17,18]. Peptides of up to at least 5 amino acid residues are transported [1,5,6]. Transport displays stereo-chemical specificity for peptides containing L-amino acid residues [5,7]. During development, uptake capacity reaches a maximum after about 3 days of germination [16], D.J. Walker-Smith and J.W. Payne, unpublished), and this pattern parallels the build up of the endogenous peptide pool resulting from storage protein hydrolysis [9]. Calculations based on en-

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dogenous levels of peptide substrates and the typical kinetic parameters of the peptide transport system indicate that peptides are likely to play at least as important a role as amino acids in the transfer of stored nitrogen from the endosperm to the embryo during germination [9,16].

Here we have studied the effects of treating scutella with protein reagents in an attempt to characterise the components of the peptide transport system. We show that SH-reagents rapidly and specifically inhibit peptide transport (relative to amino acid and glucose uptake), and that L-peptide substrates (but not D-peptides) can protect against this inactivation. Furthermore, the inhibition caused by the dithiol-specific reagent, phenylarsine oxide, is reversible by treatment with the reducing agent dithiothreitol. We conclude that peptide uptake involves a redox-sensitive, dithiol-dependent transport protein, that may exploit a similar translocation mechanism to that proposed for certain prokaryotic transport systems [11,14].

2. MATERIALS AND METHODS

2.1. *Embryo preparation*

Barley seeds (*Hordeum vulgare* L. cv. Maris otter, Winter) were wetted in 70% (v/v) aqueous ethanol for 10 s and surface-sterilized for 10 min by using sodium hypochlorite (1% w/v, available chlorine) and germinated asceptically on 1.2% (w/v) agar at 20°C for 2 days. Embryos were dissected out and stored in oxygenated 50 mM phosphate–citrate buffer (pH 3.8) at 2°C until used (up to 2 h).

2.2. *Transport assays*

Typically, 6 embryos were incubated in 1 ml of the above buffer at 20°C in a water bath, with shaking (100 strokes/min): the assay was initiated by addition of substrate, either 2 mM peptide, 2 mM amino acid or 20 mM glucose. Periodically, embryos were removed, washed with buffer, added to 0.5 ml, 5 M acetic acid in stoppered tubes and extracted on a boiling water bath for 20 min. Extracts were added to 5 ml scintillant (NE 260, Nuclear Enterprises), and left to stand overnight before being counted for radioactivity. Variations to this standard procedure are described in section 3.

2.3. *Materials*

N-Ethylmaleimide (NEM), *p*-chloromercuribenzene sulphonic acid (PCMBS) and phenylarsine oxide were obtained from Sigma (London). Dansyl aziridine was from Pierce and Warriner Ltd. Gly–[U-^{14}C]Phe (12.8 mCi.mmol^{-1}), [U-^{14}C]Leu (354 mCi.mmol^{-1}), D-[U-^{14}C]glucose (230 mCi.mmol^{-1}) and *N*-[U-^{14}C]ethylmaleimide (15 mCi.mmol^{-1}) were from the Radiochemical Centre, Amersham: Ala–Ala–[U-^{14}C]Ala (1.95 μCi.mg^{-1}) was a gift from W.J. Lloyd, Roche.

3. RESULTS

3.1. *Sensitivity of peptide transport to protein reagents*

In an attempt to locate, isolate and characterise the protein components of the peptide transport system, we sought to determine the effects on peptide uptake of pretreating scutella with various chemical reagents known to modify particular types of amino acid residues. In such studies it is important to be able to distinguish between specific effects on peptide-transport proteins and secondary, perhaps more general, reactions that might inhibit uptake indirectly. To try and resolve this difficulty we carried out two controls:

(1) We measured uptake not only of peptides but also of amino acids (leucine) and simple sugars (glucose);

(2) We performed substrate screening experiments, to see whether peptides could specifically protect against any observed inhibition.

With diethylpyrocarbonate (modifies imidazole ring of histine) both peptide and amino acid uptake were inhibited to essentially the same extent and with the same kinetics. Phenylglyoxal (modifies arginine residues) inhibited uptake of peptides more than amino acids but no substrate protection was noted. With certain reagents, e.g., *N*-ethyl-5-phenylisoxazolium-3′-sulphonate (Woodward's reagent K, for carboxyl groups), no inhibition was observed.

3.2. *Selective inhibition with thiol-reagents*

Most interestingly, rapid and specific inhibition was noted with thiol-specific reagents. Pretreatment with 5 mM *N*-ethylmaleimide for 5 min caused 90%, 35% and 32% inhibition of Gly–Phe, Leu and glucose uptake, respectively. Studies with

[^{14}C]NEM showed that it rapidly penetrated the embryo and it seemed likely that the inhibition seen with Leu and glucose (that increased slowly with time) might arise from secondary reactions consequent upon NEM uptake. When embryos were preloaded with Gly–[^{14}C]Phe and then treated with NEM as above, this did not cause significant exodus of counts, suggesting to us that NEM-treatment inhibits uptake per se rather than preventing accumulation by facilitating exodus: in contrast, treatment of embryos with 5 mM acetate (pH 3.8) caused rapid and fairly complete efflux of accumulated counts.

Treatment with *p*-chloromercuribenzene sulphonic acid 5 mM (pH 5) 20°C for 20 min gave kinetic inhibition curves similar to those with NEM. However, in contrast to NEM-induced inhibition, peptide substrates could protect against PCMBS-mediated inhibition (table 1). Both di- and tripeptides protected, but only those containing L-residues, the all D-peptide analogues, provided no protection and neither did the constituent-free L-amino acid residues. It may be that the different substrate protection effects seen with NEM and PCMBS arise because the former is a highly penetrant molecule whereas PCMBS is not and can modify SH groups on the outer surface of the scutellum only [15] (see section 4).

Table 1

Substrate protection against inhibition of peptide transport by *p*-chloromercuribenzene sulphonic acid

Substrate added	% Inhibition of peptide transport
None	72
D-Ala–D-Ala–D-Ala	71
L-Ala–L-Ala–L-Ala	31
D-Ala–D-Ala	70
L-Ala–L-Ala	37
L-Ala	75
L-Met	73
L-Leu	74

Embryos were incubated with 5 mM *p*-chloromercuribenzene sulphonic acid for 4 min at 20°C, pH 3.8, with or without added competitor (100 mM). After washing, the embryos were used in a standard assay for Gly–[U-^{14}C]Phe transport. Values represent the percentage inhibition of uptake relative to an untreated control

3.3. *Presence of redox-sensitive vicinal dithiols*

Incubation of embryos with phenylarsine oxide showed similar specific inhibition of peptide uptake (fig.1). Inhibition of uptake of Gly–[U-^{14}C]Phe and of Ala–Ala–[U-^{14}C]Ala was essentially complete (about 90%) within 5 min. In contrast to the previous SH-reagents, phenylarsine oxide is specific for vicinal or paired SH-groups, being unable to form a stable complex with single, separated SH-groups [11,19].

The inhibition of peptide transport by phenylarsine oxide or by PCMBS is reversible by subsequent treatment with a reducing agent that can

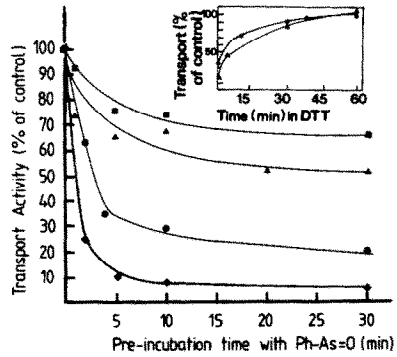

Fig.1. Inhibition of transport in barley scutellum by phenylarsine oxide. 2-Day old embryos were treated with 0.5 mM phenylarsine oxide at 20°C for the indicated times. After washing, the treated embryos were resuspended in 50 mM phosphate–citrate buffer (pH 3.8) at 20°C to measure uptake of [U-^{14}C]Leu (2 mM, 12.5 μCi.mmol^{-1}) (■—■); D-[U-^{14}C]glucose (20 mM, 1.25 μCi.mmol^{-1}) (▲—▲); Gly–[U-^{14}C]Phe (2 mM, 12.5 μCi.mmol^{-1}) (●—●); and Ala–Ala–[U-^{14}C]Ala (2 mM, 7.55 μCi.mmol^{-1}) (◆—◆). Residual transport activity is expressed as a percentage of the uptake in untreated controls. Control rates were 15, 190, 30, and 55 nmol.scutellum^{-1}.h^{-1} for Leu, glucose, Gly–Phe and Ala–Ala–Ala, respectively. Inset: Reversal by dithiothreitol of the inhibition of peptide transport by *p*-chloromercuribenzene sulphonic acid and phenylarsine oxide. 2-Day old embryos were treated with 0.5 mM phenylarsine oxide for 4 min, pH 3.8, 20°C (●—●), or with 4 mM *p*-chloromercuribenzene sulphonic acid for 30 min, pH 5, at 20°C (▲—▲). After washing, treated embryos were incubated in 10 mM dithiothreitol, pH 3.8, at 20°C, for the indicated times before being used to assay uptake of 2 mM Gly–[U-^{14}C]Phe, pH 3.8, 20°C. Rates are expressed as a percentage of the uptake in controls taken through the same incubations but without added inhibitors.

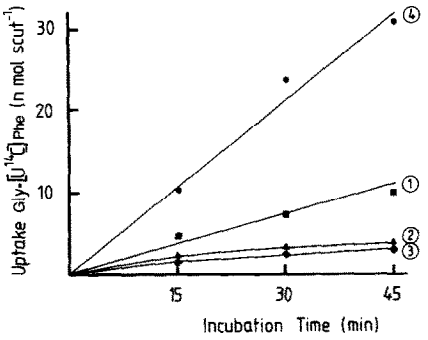

Fig.2. Procedures for selective labelling of thiol-groups involved in peptide transport. Curves represent the uptake of Gly–[U-^{14}C]Phe with time by embryos subjected to the following treatments: (1) phenylarsine oxide, *N*-ethylmaleimide, dithiothreitol; (2) *N*-ethylmaleimide, dithiothreitol; (3) phenylarsine oxide, *N*-ethylmaleimide; (4) control incubated in 50 mM phosphate–citrate buffer for 1 h, pH 3.8, 20°C. All phenylarsine oxide inhibitions were at 0.5 mM, pH 3.8, 20°C for 4 min; all *N*-ethylmaleimide inhibitions were at 5 mM, 20°C, for 3 min; and all dithiothreitol treatments were at 10 mM, for 1 h at pH 3.8, 20°C. Scutella were washed thoroughly between treatments with distilled water.

regenerate the free SH-groups. Thus, transport capacity is largely restored after 60 min incubation with dithiothreitol (fig.1, inset). Controls showed that this same treatment did not have a restorative effect upon the much smaller degree of inhibition of Leu and glucose uptake caused by PCMBS or phenylarsine oxide.

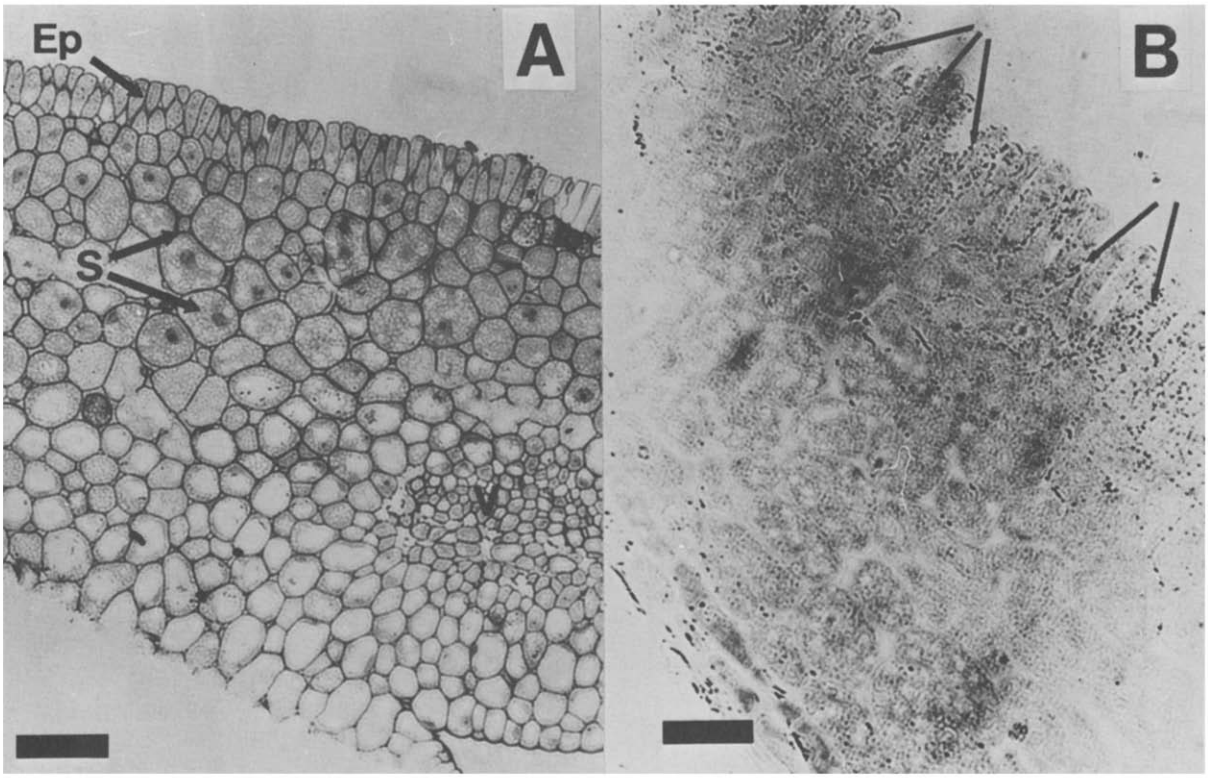

Fig.3. (A) Vertical section through barley scutellum showing elongated columnar cells forming the surface epithelium (Ep) with underlying subepithelial layers (s) and vascular tissue (v). Bar represents 90 μm. (B) Autoradiogram of scutellar tissue specifically labelled with [^{14}C]NEM as described in fig.2 and in the text. Following extraction of unbound label with 5 mM acetic acid, sections were covered with a monolayer of Ilford L4 photographic emulsion, exposed for 5 days and developed. Silver grains (arrowed), located primarily in the plasmalemma of the epithelial cells, show distribution of peptide-transport proteins. Bar represents 35 μm.

3.4. *Specific labelling of peptide transport proteins*

These observations provide a means to label selectively the SH-containing proteins to aid in identification of their location, and in their isolation. Thus, scutella are treated first with phenylarsine oxide to derivatise all vicinal dithiols, and then with NEM to react with all remaining SH groups: after washing away residual NEM the derivatized dithiols are regenerated by treatment with dithiothreitol, a procedure that restores a proportion of the peptide transport activity. Finally, the exposed vicinal dithiols are labelled with [^{14}C]-NEM, and amongst these will be those that are components of the peptide transport system: transport assays run at this stage indicate that peptide uptake has been inhibited (fig.2). A further control shows that NEM treatment alone is not reversible by dithiothreitol (fig.2). Radioautography of scutella treated in this way reveals highly selective labelling of membrane thiol sites at the endosperm surface of the plasmalemma (fig.3). The selectivity of this binding is particularly marked, for if radioautography is carried out omitting the acetic acid wash, [^{14}C]NEM is seen to penetrate throughout the tissues (not shown) and so potentially can derivatise any available protein-SH groups.

In related studies, we have used the non-penetrating, SH-specific reagent, dansyl aziridine, which forms fluorescent adducts with thiols, and found it also selectively blocks peptide transport (not shown). It seems likely, therefore, that these various SH-reagents, when used in appropriate combinations, should allow specific labelling of thiol groups on the outer and the inner surfaces of the single layer of columnar, epithelial cells that presumably are the location of the active transport systems in the scutellum [2].

4. DISCUSSION

The results indicate that peptide transport across the scutellum of germinating barley requires the participation of protein-bound SH-groups. These SH-groups are presumably located at a substrate-binding site because transportable peptide substrates can protect against the inhibition of transport caused by thiol-specific protein reagents. Protection is not afforded by D-peptides which are not transport substrates, or by free amino acids. Furthermore, extensive protection is only exercise against thiol reagents (e.g., *p*-chloromercuribenzene sulphonic acid) that cannot penetrate the plasmalemma of the surface epithelial cells, and not against penetrating reagents such as *N*-ethylmaleimide and phenylarsine oxide [19]: we infer that the transport system may have essential thiol-groups located on both the outer and the inner surfaces of the plasmalemma. The rapid and selective inhibition of peptide uptake by phenylarsine oxide shows these SH-groups to be vicinal dithiols. The inhibition caused by this reagent and by *p*-chloromercuribenzene sulphonic acid (but not by *N*-ethylmaleimide) is reversible by treatment with a reducing agent, dithiothreitol. Thus, peptide uptake appears to depend upon the action of redox-sensitive dithiol groups. In this regard, it resembles the activity of transport proteins for lactose and proline in *Escherichia coli*, systems in which transport is linked to an electrochemical proton gradient [11]. The ease with which the scutellar transport systems can be studied, coupled with the regulated developmental pattern of germination, makes this an excellent higher plant system in which to unravel molecular mechanisms of transport and to make comparison with the more widely studied microbial and mammalian systems.

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