Identification of a 23 kDa protein from maize photoaffinity-labelled with 5-azido-[7-^{3}H]indol-3-ylacetic acid

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A 23 kDa protein (p23) was identified in microsomal extracts from maize coleoptiles by photoaffinity labelling with 5-azido-[7-^{3}H]indol-3-ylacetic acid ([^{3}H]N_3IAA). Labelling of p23 was blocked by unlabelled IAA, N_3IAA, indol-3-ylbutyric acid and indol-3-yl-lactate. In addition, labelling was efficiently decreased by tryptophan, as well as by the scavenger *p*-aminobenzoic acid. Labelling was, however, not affected by synthetic auxins such as 1-naphthylacetic acid or 2,4-dichlorophenoxyacetic acid. Competition data suggest that the label was probably bound via the indole ring, and hence labelling was not specific for auxins. The 23 kDa protein was solubilized from crude microsomes by extraction with Triton X-100 and purified to homogeneity by ion-exchange, size-exclusion and reversed-phase chromatography. After electroblotting, the amino acid sequences of the p23 N-terminus as well as the several tryptic peptides were obtained. Database comparisons revealed sequence identity with a maize manganese superoxide dismutase. We conclude that photoaffinity labelling of p23 was pseudo-affinity, and therefore the binding site for IAA is not specific.

INTRODUCTION

Auxins influence a wide range of growth and developmental responses in plants (for review see Davies, 1987). Despite the well-documented physiological effects of auxins, the molecular mechanisms underlying these responses are not yet well understood. According to a widely accepted view, it is thought that auxin is recognized by specific receptor proteins which, after binding of the hormone, transmit the signal into the plant cell (Guern, 1987). The search for such receptor proteins was based for a long time on equilibrium binding of auxin to extracts from various subcellular fractions obtained from numerous plant species (for review see Palme, 1994). Although these studies resulted in identification of several auxin-binding sites, the molecular identity of most of these sites remains to be determined.

Initial biochemical characterization using equilibrium auxin-binding assays met with limited success. Recently, however, photoactivatable auxin analogues such as azido-[7-^{3}H]indol-3-ylacetic acid (N_3IAA) were successfully used to identify auxin-binding proteins. Among the proteins identified were a 40 kDa protein and a 42 kDa protein from zucchini hypocotyl, which may be involved in active auxin uptake (Hicks et al., 1989, 1993). In maize seedling plasma membranes a 23 kDa protein (pm23) and a 24 kDa protein (pm24) were found (Feldwisch et al., 1992). Soluble proteins identified by [^{3}H]N_3IAA labelling include a 60 kDa β-glucosidase from maize, an enzyme that was shown to hydrolyse cytokinin *O*-glucosides and kinetin N^3-glucoside (Campos et al., 1992; Brzobohaty et al., 1993). In addition, soluble and membrane-associated glutathione *S*-transferases from *Hyoscyamus muticus* and *Arabidopsis thaliana* were identified and biochemically characterized (Macdonald et al., 1991; Bilang et al., 1993; Zettl et al., 1994).

Jones and Venis (1989) reported photoaffinity labelling of a 22 kDa and a 24 kDa protein from maize seedlings with [^{3}H]N_3IAA. Whereas the 22 kDa protein seems to correspond to the Zm-ERabp 1 protein, a member of a now well-studied group of auxin-binding proteins, no further information is available for the 24 kDa protein.

Here we report the identification by [^{3}H]N_3IAA labelling and purification of a 23 kDa protein (p23) from maize seedlings that apparently corresponds to the 24 kDa protein identified by Jones and Venis (1989). Partial amino acid sequence analysis revealed sequence identities of p23 with a manganese superoxide dismutase (Mn-SOD).

MATERIALS AND METHODS

Radiochemicals and chemicals

5-N_3-[7-^{3}H]IAA [21.7 Ci/mmol (1 Ci = 37 GBq)] was synthesized as described by Campos et al. (1991). All chemicals were of analytic grade and purchased from Sigma (München, Germany) or Merck (Darmstadt, Germany) if not stated otherwise.

Plant material

Seeds of *Zea mays* L. (cv. mutin 240; Kleinwanzlebener Saatzucht, Einbeck, Germany) were soaked in water overnight and grown on moist cotton for 3 days in the dark at 28 °C. Coleoptiles, including the primary leaf, were harvested, frozen in liquid nitrogen and stored at −70 °C.

Photoaffinity labelling

Photoaffinity labelling was performed in the presence of dim red light as described previously (Campos et al., 1991; Feldwisch et al., 1994). Samples containing 100 μg of protein were incubated in buffer 1 (30 mM sodium citrate/citric acid, pH 5.5, 5 mM $MgCl_2$) containing 1 μM [^{3}H]N_3-IAA for 10 min on ice. Optionally, unlabelled auxins and related compounds were added for competition analysis. Photolysis was performed at 4 °C for 10 min by using a high-pressure mercury lamp (Philips HKP

Abbreviations used: IAA, indol-3-ylacetic acid; [^{3}H]N_3IAA, 5-azido-[7-^{3}H]IAA; IBA, indol-3-ylbutyric acid; ILA, indol-3-yl-lactate; 1- (or 2-) NAA, 1- (or 2-) naphthylacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; PABA, *p*-aminobenzoic acid; SOD, superoxide dismutase; PVDF, polyvinylidene difluoride.

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125 W/L). After illumination, proteins were precipitated with 10 % trichloroacetic acid to remove unincorporated radioactivity. Proteins were dissolved in SDS-loading buffer, heated to 100 °C for 5 min, and separated by SDS/PAGE (12.5 %; Laemmli, 1970). Fluorography was performed as described by Skinner and Griswold (1983). Dried gels were exposed for 4 days to pre-flashed Kodak XAR-5 films.

Purification of p23

All steps were performed at 4 °C. Coleoptiles, including the primary leaves, were homogenized with a Waring Blendor and a Polytron homogenizer (at setting 7–8) in 2.5 vol. (2.5 ml/g fresh wt.) of buffer 2 (100 mM Tris/citric acid, pH 8.0, 250 mM sucrose, 1 mM EDTA, 5 mM ascorbic acid, 0.1 mM $MgCl_2$, 4 mM diethyldithiocarbamic acid, 3.5 mg/l aprotinin, 0.1 μg/ml leupeptin). After filtration through a nylon net (135 μm), the particulate material retained in the net was re-extracted in buffer 2 (1:1, v/v). The combined homogenates were centrifuged for 10 min at 10000 ***g***. The supernatant was centrifuged at 142000 ***g*** for 40 min. The microsomal pellet obtained from 900 g of coleoptile tissue was resuspended in 200 ml of buffer 3 (10 mM Mes/NaOH, pH 5.5, 250 mM sucrose, 5 mM $MgCl_2$) containing 0.5 % Triton X-100, stirred for 1.5 h at 4 °C and subsequently centrifuged as described above. Solubilized proteins were diluted with 1 vol. of buffer 3, applied on a DEAE fast-flow column (Pharmacia; 27 ml bed volume) and eluted with 0.1 M NaCl in buffer 3. The 0.1 M NaCl eluate containing the 23 kDa protein was precipitated with $(NH_4)_2SO_4$ (75 % saturation) and applied on to a Sephacryl HR S-200 column (Pharmacia; 483 ml gel bed volume) in buffer 4 (20 mM Tris/HCl, pH 7.3, 250 mM sucrose, 100 mM NaCl). Samples of the fractions containing the 23 kDa protein were diluted with 2 vol. of solution A (0.06 % trifluoroacetic acid in water) and further purified by reversed-phase chromatography using a 4.6 mm × 250 mm C_4 column (VYDAC Separation Group, U.S.A.). Proteins were eluted with a linear gradient from 0 to 100 % Solution B (0.056 % trifluoroacetic acid in 80 % acetonitrile/water) in 60 min and analysed by SDS/PAGE.

Protein sequence analysis

Proteins separated by SDS/PAGE were electroblotted on to polyvinylidene difluoride (PVDF) membranes (Millipore. Eschborn, Germany) in 50 mM Tris/50 mM boric acid, pH 8.3. Proteins were revealed after staining for 40 s with Amido Black [0.1 % in 45 % methanol (h.p.l.c. grade)/water] and destaining with water. For proteolytic digestion with trypsin, protein-containing membrane pieces were excised and incubated in 100 mM Tris/HCl, pH 8.0, with 1 μg of trypsin (Boehringer, Mannheim, Germany) for 4 h at 37 °C. Tryptic peptides were recovered after washing the excised PVDF pieces once with 100 μl of 80 % formic acid and four times with 100 μl of water, and then separated on a VYDAC C_{18} reversed-phase column (4.6 mm × 250 mm). Peptides were eluted at a flow rate of 0.4 ml/min with a linear gradient of 0–37 % solvent B in solvent A for 61 min, 37–75 % B for 32 min, and 75–100 % B for 10 min. Solvent A was aq. 0.06 % trifluoroacetic acid, and solvent B was 80 % (v/v) acetonitrile in aq. 0.056 % trifluoroacetic acid. Peptides were detected at 214 nm by using a diode array detector to allow peak purity analysis (HP-1090 HPLC; Hewlett–Packard Waldbronn, Germany). Peptides were collected by hand and used directly for sequence analysis by automated Edman degradation in an Applied Biosystems 477A sequenator equipped with an on-line phenylthiohydantoin amino acid analyser (120A). Both sequenator and analyser were operated as described by the manufacturer. For N-terminal sequence analysis, excised PVDF pieces (2 mm × 5 mm) were mounted directly in the reaction chamber of the protein sequenator.

Immunological techniques

Polyclonal antisera were raised in rabbits against recombinant ZmERabp1 (T. Hesse and K. Palme, unpublished work) by the procedure described by Catty and Raykundalia (1988). For immunostaining of Western blots, a secondary goat anti-rabbit antibody coupled to alkaline phosphatase was used, as described by Blake et al. (1984).

RESULTS

Photoaffinity labelling of maize microsomes with [^{3}H]N_3IAA

Crude microsomes were isolated from etiolated maize coleoptiles at day 3 after imbibition. Microsomal proteins were solubilized with 0.5 % Triton X-100, and proteins were bound to a DEAE-Sepharose column. After extensive washing of the column, proteins were eluted with 0.1 M NaCl, concentrated by precipitation with $(NH_4)_2SO_4$ (75 % satn.) and loaded on to an S-200 column. All fractions obtained by size-exclusion chromatography were analysed by photoaffinity labelling and immunostaining (Figure 1). Using photoaffinity labelling, we identified four proteins with molecular masses of 60 kDa (p60), 58 kDa (p58), 23 kDa (p23) and 22 kDa (ZmERabp1). Results for p60, p58 and p22 have been published previously (Palme et al., 1990; Campos et al., 1992). The identity of p22 was identified after electroblotting onto PVDF and immunostaining with the polyclonal antibody PAB130 raised against recombinant ZmERabp1. We observed that, of all the proteins, only the 22 kDa (p22) was detected by PAB130, indicating that this protein corresponds to the maize abp1 protein (ZmERabp1) (Figure 1c). The protein of 21 kDa seen in Figure 1(c) apparently corresponds to a degradation product of ZmERabp1. When we extracted our micro-

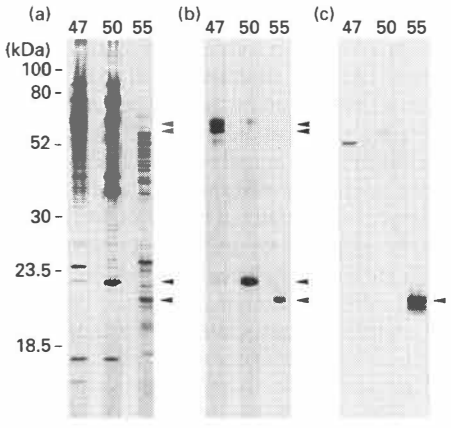

Figure 1 Identification of auxin-binding proteins by using [^{3}H]N_3IAA and polyclonal antibody PAB130

Proteins solubilized with Triton X-100 from maize microsomes were partially purified by anion-exchange chromatography and gel filtration (S-200). Fractions were labelled with 1 μM [^{3}H]N_3IAA at 4 °C and separated by SDS/PAGE. (**a**) Silver-stained gel; (**b**) fluorography of gel shown in (**a**); (**c**) Western blot of gel shown in (**a**). Arrowheads indicate p60, p58, p23 and p22 (ZmERabp1). Molecular size markers are indicated at the left, and fraction numbers (from S-200) on the top. Calibration of the S-200 column was performed by using aldolase (158 kDa), BSA (66 kDa), ovalbumin (43 kDa) and chymotrypsin (25 kDa) as molecular size markers.

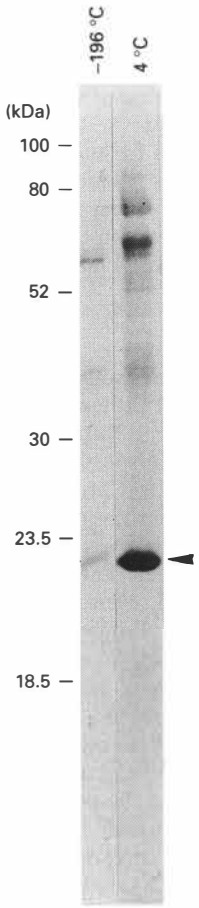

Figure 2 Labelling of p23 with [^{3}H]N_3IAA

p23 was partially purified by ion-exchange and size-exclusion chromatography, followed by labelling with 1 μM [^{3}H]N_3IAA at 4 °C or −196 °C. Proteins were separated by SDS/PAGE. Labelled proteins were revealed on a pre-flashed Kodak X-Omat AR-5 film by fluorography (5 days at −70 °C).

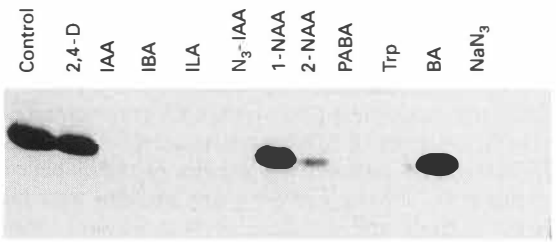

Figure 3 Competition of [^{3}H]N_3IAA labelling with auxins and relevant analogues

Partially purified p23 was labelled with 1 μM [^{3}H]N_3IAA at 4 °C with or without 1 mM competitor and separated by SDS/PAGE. Fluorography was for 4 days at −70 °C, using a pre-flashed Kodak X-Omat AR-5 film. Other abbreviation: BA, benzoic acid.

somal membranes with acetone, a procedure well known to release auxin-binding proteins from the lumen of the endoplasmic reticulum, we obtained, after DEAE-Sepharose and size-exclusion chromatography followed by [^{3}H]N_3IAA labelling, essentially the same results (not shown).

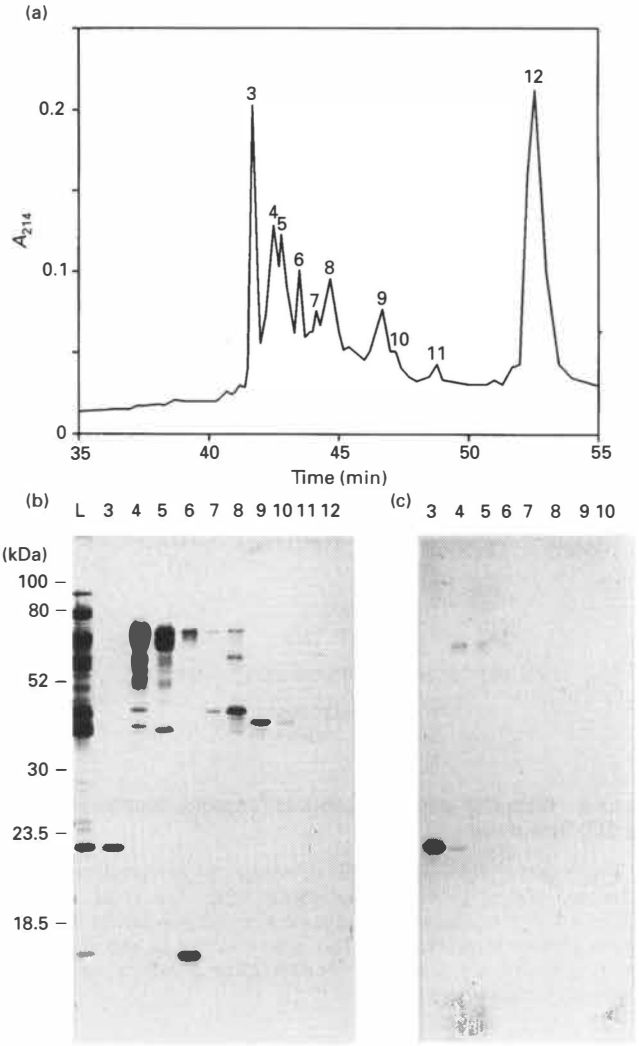

Figure 4 Purification of p23 by reversed-phase chromatography

p23 was purified to homogeneity by chromatography on a reversed-phase column (VYDAC C_4). Proteins were eluted by a linear acetonitrile gradient ranging from 0 to 100% B in 60 min and analysed by SDS/PAGE. (**a**) Elution profile; (**b**) silver-stained SDS gel; (**c**) fluorograph of (**b**), 4-day exposure at −70 °C. Lanes 3–12 correspond to the peaks eluted from the reversed-phase C_4 column; L, protein fraction loaded on the C_4 column.

Temperature-dependence and competition of p23 photoaffinity labelling

The specificity of p23 labelling with [^{3}H]N_3IAA was assessed by labelling: (i) at different temperatures, (ii) in the presence of competitors and (iii) in the presence of a scavenger. As shown in Figure 2, p23 was strongly labelled at 4 °C, whereas labelling at −196 °C was significantly decreased. For competition analysis, S-200 fractions containing p23 were incubated at 4 °C with [^{3}H]N_3IAA and various unlabelled auxins or relevant analogues before illumination (Figure 3). [^{3}H]N_3IAA labelling of p23 was inhibited by auxins such as IAA, indol-3-ylbutyric acid (IBA), indol-3-lactate (ILA) and unlabelled N_3IAA. It was inhibited by the amino acid tryptophan and partially inhibited by the weak auxin analogue 2-naphthylacetic acid (2-NAA). We did not observe competition of photolabelling by the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthylacetic acid (1-NAA), or by benzoic acid. Our data suggest that only molecules with an

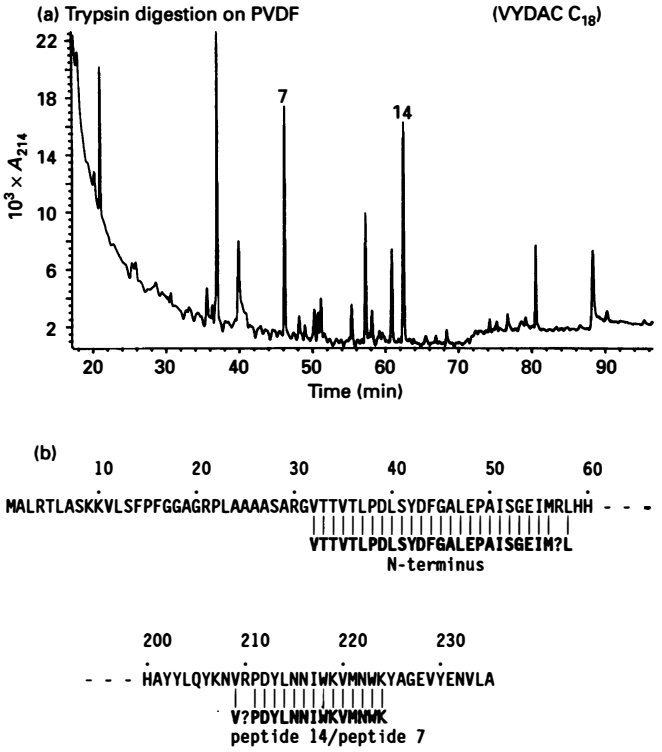

Figure 5 Separation of tryptic peptides and sequence identities of p23 with Mn-SOD from maize

(**a**) Tryptic peptides of p23 were obtained as described in the Materials and methods section and separated by reversed-phase chromatography on a VYDAC C_{18} column (250 mm × 4.5 mm). Peptides were sequenced by automated Edman degradation. (**b**) Sequence identities of p23 with MN-SOD from maize. The N-terminal sequence and sequences of peptides 7 and 14 (in bold letters) are compared with the maize SOD sequence. Numbering is according to White and Scandalios (1988).

indole ring are able to compete efficiently for [^{3}H]N_3IAA binding. Interestingly [^{3}H]N_3IAA labelling of p23 was also strongly inhibited by NaN_3.

To analyse the labelling specificity further, we tested the influence of a scavenger (*p*-aminobenzoic acid, PABA). Scavenger bind excess photogenerated reactive nitrenes, thereby preferentially restricting labelling to the binding site of the target protein. As shown in Figure 3, [^{3}H]N_3IAA labelling of p23 was completely inhibited when the labelling reaction was performed in the presence of 1 mM PABA. This suggests that labelling of p23 is probably due to pseudo-photoaffinity labelling.

Partial amino acid sequence analysis of p23 reveals homology to a maize Mn-SOD

After partial purification of p23 by DEAE-Sepharose and S-200 chromatography, p23 was labelled with [^{3}H]N_3IAA and further analysed by reversed-phase chromatography (Figure 4). Labelled proteins were bound to a VYDAC C_4 column and eluted with a linear acetonitrile gradient. Each fraction was analysed by SDS/PAGE. One half of the gel was stained with silver, and the other half was subjected to fluorography. As shown in Figures 4(b) and 4(c), [^{3}H]N_3IAA-labelled p23 was eluted as homogeneous protein in fraction 3.

For protein sequence analysis, purified p23 was separated by SDS/PAGE and electroblotted on to PVDF. The N-terminal amino acid sequence of p23 was determined directly on Amido-Black-stained PVDF. Internal amino acid sequence information was obtained after tryptic cleavage of Amido-Black-stained PVDF containing p23. Released peptides were separated on a VYDAC C_{18} column (Figure 5a) and the numbered peaks were used for sequence analysis by automated Edman degradation. The amino acid sequences obtained are listed in Figure 5(b). Homology searches (PIR protein data base) revealed identity of all amino acid residues determined with those of a maize Mn-SOD (White and Scandalios, 1988).

DISCUSSION

Photoaffinity labelling is a method to introduce covalent chemical tags into the active sites of protein molecules. This method has contributed greatly to identification and structural studies of numerous receptors and transport proteins since its introduction (Bayley and Knowles, 1977; Chowdry and Westheimer, 1979). Upon photolysis, a light-sensitive group of a photoaffinity ligand decomposes and covalently labels a binding polypeptide. When covalently labelled, the relevant proteins can be identified, isolated and characterized, even under denaturing conditions. The ideal properties of a photoactivatable group are chemical stability before photoactivation, rapid and complete photolysis at an appropriate wavelength, and a very high reactivity of the photogenerated species. However, none of the currently available photolabile groups meets all the requirements, and, consequently, careful consideration must be given to the nature of the group, its chemical properties and the possible artifacts that could be produced. Azides are a widely used light-sensitive group in photolabelling research, because, upon illumination, the generated nitrene can react rapidly and relatively unselectively with its immediate environment. Nitrenes may have half-lives in the order of milliseconds, and insert in a variety of bonds, including aliphatic C-H bonds. Rearrangements of bonds during the labelling reaction have been noted; however, these potential problems do not diminish the value of this approach when reactions and their products are carefully controlled.

Since photoaffinity labelling has been a powerful tool for identification of numerous ligand-binding proteins, synthetic phytohormone analogues carrying photolabile groups had been synthesized as early as 1975 (Leonard et al., 1975). Azido-IAA derivatives were synthesized by Melhado et al. (1982) and demonstrated to have auxin activity (Melhado et al., 1984; Jones et al., 1984a). After incubation of maize seedling extracts with azido-IAA and subsequent photolysis, covalent complexes were formed with a number of cellular constituents, as demonstrated by SDS/PAGE and radioactivity profiles of radiolabelled proteins (Jones et al., 1984b). Far too many proteins were labelled in these initial trials, and therefore labelling was not considered to be specific. That unspecific labelling may occur is well known, and has been observed, for example, for the labelling of leukotriene-binding sites (Falk et al., 1989). However, high background labelling can be decreased by direct photoaffinity labelling in the deep-frozen state, and so it was not surprising that labelling of auxin-binding proteins with [^{3}H]N_3IAA occurred with considerable specificity when labelling was performed at −196 °C (Hicks et al., 1989).

A variety of putative auxin-binding proteins have meanwhile been identified by labelling with [^{3}H]N_3IAA and partially characterized (Bilang et al., 1993; Campos et al., 1991, 1992; Feldwisch et al., 1992; Hicks et al., 1989, 1993; Jones and Venis, 1989; Macdonald et al., 1991; Zettl et al., 1994). Among the proteins labelled were ZmERabp1, the best studied auxin-binding protein (Jones and Venis, 1989). This 22 kDa protein was labelled

together with another, as yet uncharacterized, 24 kDa protein, by using a purification scheme for ZmERabp1 developed by Napier et al. (1988). Using the same purification strategy we identified in maize coleoptile extracts four proteins with molecular masses of 60 kDa (p60), 58 kDa (p58), 23 kDa (p23) and 22 kDa (ZmERabp1) in S-200 column eluates by photoaffinity labelling with [^{3}H]N_3IAA. The labelling patterns that we observed for p23 and ZmERabp1 were similar to those published by Jones and Venis (1989). It therefore seems highly likely that p23 and the 24 kDa protein identified by Jones and Venis (1989) are identical. The small difference in molecular mass (1 kDa) could be due to different molecular-mass markers used to calculate the apparent molecular masses of these proteins. Our antibodies directed against ZmERabp1 did not recognize p23, similar to the findings of Jones and Venis (1989). As revealed by partial amino acid sequencing and the availability of cDNA sequences, p23 was not related to pm23, a protein identified previously by us in maize plasma membranes (Feldwisch et al. 1992; J. Feldwisch and K. Palme, unpublished work). In addition, we noted that optimal labelling temperature, the competition of photolabelling with synthetic unlabelled auxin analogues and the solubilization characteristics from membrane vesicles with detergents were different for p23 and pm23.

The purpose of this study was to determine the identity of the p23 protein. We purified p23 to homogeneity by anion-exchange, size-exclusion and reversed-phase chromatography. The protein was eluted from the S-200 column just before BSA (66 kDa), which was used for calibration, indicating that this protein forms tri- or tetra-mers under non-denaturing conditions (results not shown). Partial amino acid sequence analysis revealed that all amino acid residues determined for p23 corresponded exactly to those of a previously published manganese superoxide dismutase (White and Scandalios, 1988). Since p23 was detected by [^{3}H]N_3IAA labelling, we performed experiments to distinguish between specific and unspecific labelling. Specific photoaffinity labelling of p23 (Mn-SOD) was assessed by labelling: (i) at different temperatures, (ii) in the presence of competitions and (iii) in the presence of a scavenger. [^{3}H]N_3IAA labelling of p23 was optimal at 4 °C, but was drastically decreased at −196 °C. Competition analysis revealed that the indole derivatives IAA, IBA, ILA and tryptophan efficiently inhibited [^{3}H]N_3IAA labelling of p23. 2-NAA or 1-NAA, 2,4-D and benzoic acid, however, decreased the [^{3}H]N_3IAA labelling of p23 only slightly or not at all, indicating that binding of [^{3}H]N_3IAA to p23 is apparently specific for the indole ring. In addition, [^{3}H]N_3IAA labelling of p23 is completely inhibited in the presence of the scavenger PABA. These results suggest that [^{3}H]N_3IAA labelling of p23 (Mn-SOD) is not specific, but may be due to pseudo-photoaffinity labelling.

The three-dimensional structure of an iron-SOD complexed with the competitive inhibitor azide was published by Stoddard et al. (1990). It was shown that azide is bound at the open coordination position of the iron. Since iron-SOD and Mn-SOD are structural homologues, with identical amino acids binding the cofactor (Fe or Mn) (Parker and Blake, 1988), the binding of azide by SOD within the binding site could offer an explanation for the pseudo-photoaffinity labelling of the maize Mn-SOD by [^{3}H]N_3IAA.

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