Purification and characterization of protein disulphide-isomerase from the unicellular green alga *Chlamydomonas reinhardtii*

A 120 kDa dimer antigenically distinct from the vertebrate enzyme

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Protein disulphide-isomerase (PDI) has been isolated from the unicellular green alga *Chlamydomonas reinhardtii* and purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration and DEAE-Sephacel, hydroxyapatite and f.p.l.c. chromatography. The active algal enzyme is a 120 kDa dimer with a subunit molecular mass of 60 kDa when determined by SDS/PAGE. Although similar in size to the previously isolated vertebrate PDIs, the algal enzyme is antigenically distinct, polyclonal antibodies against the algal PDI showing no cross-reactivity with the vertebrate enzyme on immunoblots, and vice versa. The anti-(algal PDI) antiserum did not inhibit algal PDI activity, and *C. reinhardtii* PDI could be immobilized on anti-PDI-Protein A-Sepharose in active form. In contrast with the situation in vertebrates, where PDI functions as a subunit of prolyl 4-hydroxylase, the *C. reinhardtii* PDI is not associated with the algal prolyl 4-hydroxylase.

INTRODUCTION

Disulphide bonds stabilize the tertiary and quaternary structure of many extracellular proteins, such as procollagens, albumins, immunoglobulins, enzymes and hormones. The formation of these bonds is catalysed by protein disulphide-isomerase (PDI, EC 5.3.4.1), an enzyme that has been purified from several vertebrates (see Forster & Freedman, 1984; Hillson *et al.*, 1984; Koivu & Myllylä, 1987; Freedman *et al.*, 1988, 1989; Freedman, 1989). Vertebrate PDI is a dimer consisting of two identical subunits which vary in molecular mass, when estimated by SDS/PAGE, from 56 to 61 kDa, depending upon the species of origin. The cloned cDNA sequences encode a protein of 491 amino acids and a signal sequence of 17 amino acids from rat (Edman *et al.*, 1985) and human (Pihlajaniemi *et al.*, 1987), and a slightly larger protein of 493 amino acids and a signal sequence of 22 amino acids from chicken (Kao *et al.*, 1988; Parkkonen *et al.*, 1988).

Consistent with its function of disulphide-bond rearrangement in secretory proteins, PDI is a microsomal enzyme, loosely associated with the luminal surface of the endoplasmic-reticulum membrane (Bulleid & Freedman, 1988; Freedman, 1989). The *N*-terminal sequence of the protein is characterized by a hydrophobic signal peptide, indicating that PDI is transported into the endoplasmic reticulum, and the *C*-terminal sequence, Lys-Asp-Glu-Leu, has been proposed by Munro & Pelham (1987) to be sufficient to retain the protein in this organelle. The active site for disulphide isomerization has been proposed to be the Cys-Gly-His-Cys sequence, which occurs twice in PDI, and shows a high identity with the Cys-Gly-Pro-Cys sequence in prokaryotic and eukaryotic (both plant and animal) thioredoxins (Hillson & Freedman, 1979; Edman *et al.*, 1985; Parkkonen *et al.*, 1988).

It has recently been found that PDI is a multifunctional protein, serving also as the β -subunit of prolyl 4-hydroxylase (Koivu *et al.*, 1987; Pihlajaniemi *et al.*, 1987). The catalytic mechanism of the hydroxylation reaction of peptidyl-proline,

however, does not require PDI activity (Myllylä *et al.*, 1989). Molecular cloning and nucleotide-sequencing data suggest that PDI may also serve as a cellular thyroid-hormone-binding protein (Cheng *et al.*, 1987; Yamauchi *et al.*, 1987) and iodothyronine 5'-monodeiodinase (Boado *et al.*, 1988). Furthermore, a chick glycosylation-site-binding protein that is a component of oligosaccharidyltransferase (Geetha-Habib *et al.*, 1988) possesses a high sequence identity with PDI (for reviews on the various functions, see Freedman, 1989; Kivirikko *et al.*, 1989, 1990).

The vertebrate PDI is also known to rearrange disulphide bonds in plant proteins *in vitro* such as soya-bean trypsin inhibitor, ricin and other plant toxins, and γ -gliadin, a wheat (*Triticum aestivum*) storage protein (Steiner *et al.*, 1965; Barbieri *et al.*, 1982; Bulleid & Freedman, 1988). An enzyme with PDI activity has been detected in the endoplasmic reticulum of wheat (Grynberg *et al.*, 1978; Roden *et al.*, 1982). Although the subcellular location and catalytic properties of the plant PDI appear to be similar to those of the vertebrate enzyme (Freedman *et al.*, 1984), it is not yet clear how much of the protein has been conserved. To date, no non-vertebrate PDI has been purified. Furthermore, it is not currently known whether the multiple functions ascribed to the PDI protein are limited to animals. We have purified and characterized PDI from the unicellular green alga *Chlamydomonas reinhardtii* in order to compare its properties with those of the vertebrate enzyme and to determine whether PDI is a functional component of the algal prolyl 4-hydroxylase.

MATERIALS AND METHODS

Materials

RNA and RNAase (Type XII-A) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Randomly cross-linked RNA was prepared as described previously (Myllylä & Oikarinen, 1983). Alkaline phosphatase-conjugated goat anti-(rabbit IgG) and alkaline phosphatase colour reagents were obtained from Bio-

Abbreviation used: PDI, protein disulphide-isomerase.

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Rad Laboratories (Richmond, CA, U.S.A.). Protein A-Sepharose CL 4B was from Pharmacia. *Chlamydomonas reinhardtii* (wall-less strain cw 92) was obtained from the Chlamydomonas Genetics Center, Duke University, Durham, NC, U.S.A. The cells were grown under constant illumination in the phosphate-buffered medium described previously (Solter & Gibor, 1977) supplemented with sodium acetate (1 g/litre).

Purification of PDI

Cells (32 litres; approx. 10^9 cells/litre) were harvested by centrifugation (1000 g, 5 min) and lysed in 100 ml of solubilization buffer [100 mM-sodium phosphate buffer (pH 7.5)/100 mM-NaCl/5 mM-EDTA/0.1% Triton X-100]. After incubation at 4 °C for 12–14 h the mixture was centrifuged (15000 g, 20 min) and the supernatant adjusted to 85% -satd. $(\text{NH}_4)_2\text{SO}_4$ at 4 °C. After overnight precipitation, the solution was centrifuged and the pellet resuspended in 25 ml of citrate buffer (25 mM-sodium citrate/100 mM-NaCl/1 mM-EDTA, pH 6.2) and dialysed extensively against this buffer. The enzyme solution was then frozen at -70 °C. After thawing, insoluble material was removed by centrifugation (15000 g, 20 min).

The supernatant was then applied to a column of DEAE-Sephacel (Pharmacia) (15 cm $\times$ 2.5 cm) equilibrated with the above dialysis buffer. PDI activity was bound to the column and was eluted with the same buffer containing NaCl in a linear gradient from 100 to 500 mM (total vol. 800 ml). The fractions containing PDI activity were pooled, concentrated to 8 ml and applied to a Bio-Gel A 1.5m (Bio-Rad) column (82 cm $\times$ 2.5 cm) equilibrated in 50 mM-sodium phosphate buffer, pH 7.2. The column was calibrated with standards of known M_r .

The fractions containing PDI activity were pooled and applied to a 2 cm $\times$ 2.5 cm hydroxyapatite column (Bio-Gel HTP, Bio-Rad) equilibrated in 50 mM-sodium phosphate buffer, pH 7.2. The enzyme bound to the column and was eluted at room temperature with a linear gradient of sodium phosphate buffer from 50 to 300 mM (total vol. 500 ml). The active fractions were pooled, concentrated to 6 ml, and frozen at -70 °C. Aliquots (1.5 ml) of the hydroxyapatite-purified PDI were dialysed against water and applied to a PRORPC HR 5/2 column, equilibrated in 0.1% (v/v) trifluoroacetic acid, and reverse-phase chromatography was carried out with a Waters Associates f.p.l.c. 650 system. The protein was eluted in a linear gradient (0–90%) of acetonitrile.

Assays

PDI activity was assayed by a method involving measurement of the rate of regeneration of incorrectly disulphide-linked RNAase to the native, active form. The extent of RNAase reactivation was determined by estimating the degradation of RNA, monitoring the change in A_{260} relative to A_{280} on a spectrophotometer in the dual-wavelength mode (Ibbetson & Freedman, 1976). The incubations were carried out in a final volume of 100 μ l containing 10 μ g of randomly cross-linked RNAase, 2 μ M-dithiothreitol, 1 mM-EDTA and 50 mM-Tris/HCl, pH 7.5 (30 °C). The enzyme reaction was allowed to proceed for 15 min. The re-activation of randomly cross-linked RNAase was found to be linear for at least 15 min with the enzyme concentrations used here. Enzyme units were as defined by Ibbetson & Freedman (1976).

Preparation of antibodies

A rabbit was immunized with *C. reinhardtii* PDI injected subcutaneously at weekly intervals. The first injection, consisting of 18 μ g of PDI mixed with Freund's complete adjuvant, was followed by booster injections, consisting of 18 μ g of PDI mixed

with Freund's incomplete adjuvant. After 8 weeks, serum was collected and stored at -20 °C (see Koivu & Myllylä, 1987). Polyclonal antibody to bovine PDI, purified by antigen affinity chromatography, was a gift from Dr. Susan Murant, University of Kent, Canterbury, Kent, U.K.

Immunoblots

Purified *C. reinhardtii* PDI and chicken prolyl 4-hydroxylase were subjected to reducing SDS/PAGE and transferred to nitrocellulose membranes as described by Towbin *et al.* (1979). The nitrocellulose membranes were incubated in primary polyclonal antibodies diluted with 1% (w/v) BSA in Tris-buffered saline [0.02 M-Tris/HCl (pH 7.5)/0.5 mM-NaCl]. Bound antibody was detected with alkaline-phosphatase-conjugated goat-anti-rabbit IgG [diluted 1:3000 in 1% BSA in phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4)] and developed with alkaline phosphatase colour reagent.

Protein A affinity chromatography

Protein A-Sepharose (70 mg) was equilibrated in phosphate-buffered saline and poured into two small columns. Antiserum (1 ml) or pre-immune serum (1 ml) was applied to each column and washed extensively with phosphate-buffered saline to remove non-bound serum components. The bound rabbit IgG was eluted with citrate/phosphate buffer (50 mM-citric acid/27 mM- Na_2HPO_4 , pH 3) and then dialysed against phosphate-buffered saline. The eluted immune and pre-immune IgG were added to separate tubes containing 70 mg of Protein A-Sepharose (equilibrated in phosphate-buffered saline) and aliquots of partially purified *C. reinhardtii* PDI. The mixtures were shaken gently for 16 h at 4 °C and then poured into two small columns and washed with phosphate-buffered saline to remove non-bound protein. The bound antigen and rabbit IgG were eluted with citrate/phosphate buffer and the fractions (0.2 ml) immediately neutralized by the addition of 3.5 mg of solid Tris base.

Other assays

SDS/PAGE was carried out under reducing conditions, with a 4.5% stacking and 8% separating gels (Laemmli, 1970). Proteins were detected by staining with Coomassie Brilliant Blue R-250. The protein content of the chromatography fractions was measured by peptide absorbance at 230 nm. The protein content of the enzyme preparations was assayed by the method of Bradford (1976).

Fig. 1. Purified *C. reinhardtii* PDI separated by SDS/PAGE

PDI (4 μ g), purified as described in the Materials and methods section, was applied to an SDS/8%-(w/v)-polyacrylamide gel, electrophoresed, and stained with Coomassie Blue. M_r standards are shown at the left.

Fig. 2. Purification of *C. reinhardtii* PDI by DEAE-Sephacel (a) and hydroxyapatite chromatography (b) and by reverse-phase f.p.l.c.

(a) $(\text{NH}_4)_2\text{SO}_4$ (85% satn.)-precipitated proteins (252 mg) were applied to a DEAE-Sephacel column. Fractions were collected and assayed for protein (A_{230}). PDI activity was eluted with a linear gradient of NaCl. (b) The active fractions obtained after Bio-Gel A 1.5M chromatography were pooled and (3.8 mg) applied to a hydroxyapatite column. Fractions were analysed for protein (A_{230}). PDI activity was eluted with a linear gradient of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.5. (c) The active fractions obtained after hydroxyapatite chromatography were pooled, concentrated and dialysed against water; 10 μg was applied to a reverse-phase PRORPC HR 5/2 column. Proteins, determined by their A_{215} , were eluted with a linear gradient of acetonitrile.

RESULTS

Isolation of PDI from *C. reinhardtii*

Purification of PDI from *C. reinhardtii* by ion-exchange chromatography, gel filtration, hydroxyapatite chromatography and reverse-phase f.p.l.c. yielded a protein migrating as a single band, M_r 60000, on SDS/PAGE (Fig. 1). As Table 1 shows, 3.2×10^{10} cells yielded about 40 μg of pure PDI. PDI activity was eluted from DEAE-Sephacel at an NaCl concentration of 0.2–0.25 M (Fig. 2a) and from hydroxyapatite at a sodium phosphate concentration of 0.15–0.18 M (Fig. 2b). When applied to the reverse-phase f.p.l.c. column in the absence of salts, PDI was eluted between 50 and 55% (v/v) acetonitrile (Fig. 2c).

The specific activity of the PDI after hydroxyapatite chromatography, purified 877-fold, was 333 units/mg, corresponding approximately to the specific activity of purified PDI reported from ox liver (Hawkins & Freedman, 1976). Higher specific activities are found for PDI purified from chick embryos (Koivu & Myllylä, 1986) and from bovine liver (Lambert & Freedman, 1983). As the activity assay measures the re-activation of incorrectly disulphide-linked RNAase, it should be considered that the condition of this substrate as well as the PDI activity are both reflected in the final specific activities calculated. Preparations of the incorrectly disulphide linked ribonuclease vary in their ability to be re-activated, and this appears to contribute to the wide range of specific activities obtained for the purified PDI.

After f.p.l.c. the fractions containing the two protein peaks were evaporated to dryness and then resuspended in 50 mM-Tris/HCl, pH 7.5, and assayed for PDI activity. The major protein peak contained detectable enzyme activity (approx. 1% of the original activity), indicating that this peak is PDI and, furthermore, that the algal enzyme shows some stability under acidic conditions.

Properties of algal PDI

The M_r of the active algal PDI, determined by gel filtration, was about 120000 (115000–125000) (Fig. 3). This suggests that the enzyme is a homodimer of two M_r -60000 subunits. Dimer M_r values ranging from 107000 to 140000 have been reported previously for the vertebrate enzyme (Lambert & Freedman, 1983; Brockway & Freedman, 1984), and an M_r of 104000 for the active PDI from wheat was reported by Freedman *et al.* (1984).

The *C. reinhardtii* PDI showed considerable heat-stability. Heating the enzyme, partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, to 50 °C for 15 min caused no detectable loss in activity. Heating under similar conditions to 57 °C caused a decrease in activity of 25%, and further heating to 62 °C reduced the PDI activity to 50% of that of the unheated sample (results not shown). Vertebrate PDIs have previously been shown to exhibit relative heat-stability (Carmichael *et al.*, 1977; Lambert & Freedman, 1983). The pH and temperature optima for the algal PDI were 7.4–7.6 and 28–30 °C respectively (results not shown), which agree with those reported for the vertebrate enzymes

Table 1. Purification of PDI from *C. reinhardtii*

Purification step	Protein (mg)	PDI activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Cell lysate	1080	420	0.39	100	—
$(\text{NH}_4)_2\text{SO}_4$ precipitation (85% satn.)	252	270	1.07	64	3
DEAE-Sephacel chromatography	13.6	68	5.0	16	13
Bio-Gel A 1.5 m chromatography	3.8	61.7	14.4	15	38
Hydroxyapatite chromatography	0.04	13.7	333	3.2	877

Fig. 3. Determination of the M_r of active *C. reinhardtii* PDI by gel-filtration chromatography

Partially purified PDI (5 ml, containing 50 mg of protein) was applied to a Bio-Gel A 1.5m column. The elution positions of M_r standards are shown (●): 1, Blue Dextran; 2, BSA; 3, ovalbumin; 4, cytochrome c; 5, Phenol Red. The elution position of PDI activity is indicated by the arrow.

Fig. 4. Immunoblots of algal and vertebrate proteins stained with antiserum against *C. reinhardtii* and anti-bovine PDI

(a) Immunoblot of purified algal PDI (1 μ g) incubated with rabbit antiserum (1:2000 dilution) against algal PDI, followed by alkaline-phosphatase-conjugated anti-(rabbit IgG) and alkaline phosphatase colour reagents. Positions of M_r standards are shown on the left. (b) Coomassie Blue-stained *C. reinhardtii* proteins (50 mg) precipitated by 85% satd. $(\text{NH}_4)_2\text{SO}_4$ and separated by SDS/PAGE (lane 1) and an immunoblot of an identical lane stained with anti-(*C. reinhardtii* PDI) (lane 2). Position of M_r standards are shown on the left. (c) Immunoblot of purified algal PDI (2 μ g) (lane 1) and chick-embryo prollyl 4-hydroxylase (4 μ g) (lane 2) stained with a polyclonal antibody against bovine PDI. Positions of M_r standards are shown on the left.

Fig. 5. Protein A-Sepharose chromatography

(a) Partially purified algal PDI was immobilized on anti-PDI-Protein A-Sepharose and the matrix was assayed for PDI activity as described in the Materials and methods section. Trace 1 shows the absorbance change obtained in the absence of added enzyme, and trace 2 shows that obtained with 10 μ l of the matrix. (b) Aliquots of *C. reinhardtii* PDI were applied to Protein A-Sepharose antisera affinity columns containing anti-(*C. reinhardtii* PDI) antiserum (●) or preimmune serum (○) and eluted at pH 3 as described in the Materials and methods section. The arrow indicates the start of elution.

(Hillson & Freedman, 1980; Brockway & Freedman, 1984; Koivu & Myllylä, 1986).

Characterization of antibodies against algal PDI

The rabbit antiserum against the *C. reinhardtii* PDI recognized the purified PDI on immunoblots (Fig. 4a) and showed high specificity, recognizing only one 60000- M_r protein band on immunoblots when *C. reinhardtii* proteins, precipitated by 85% satd. $(\text{NH}_4)_2\text{SO}_4$, were separated by SDS/PAGE (Fig. 4b). No bands were detectable when similar blots were incubated with preimmune serum at the same dilution (results not shown). The antiserum did not cross-react with the β -subunit of prollyl 4-hydroxylase (PDI) from chick embryos (results not shown). Polyclonal antibodies against bovine PDI showed no cross-reactivity with the algal PDI (Fig. 4c).

The antiserum against *C. reinhardtii* PDI did not inhibit the algal PDI activity, no inhibition being found when up to 10 μ l of antiserum was added to the reaction mixture. The absence of inhibition of PDI activity indicated that the antibodies are not against the PDI active site. Similarly, in previous studies, various antibodies against vertebrate PDI did not inhibit the activity of human PDI (Koivu *et al.*, 1987).

The antiserum against *C. reinhardtii* PDI contained IgG molecules which could specifically retard the algal enzyme on Protein A-Sepharose chromatography. Furthermore, the PDI-anti-PDI complex bound to Protein A-Sepharose showed PDI activity (Fig. 5a), confirming that the antibody is not directed against the active site of PDI. Elution of the Protein A-Sepharose immuno-affinity column at low pH resulted in the release of PDI activity (Fig. 5b). The presence of enzyme activity in the eluate after neutralization confirms that the algal PDI is relatively stable under acid conditions. No detectable PDI activity could be eluted at low pH from a similar column prepared with preimmune serum.

DISCUSSION

In the present study we report the purification, for the first time, of PDI from a plant, the unicellular green alga *Chlamydomonas reinhardtii*. The presence of a PDI in green algae suggests that the formation and rearrangement of disulphide

bonds in secreted proteins is a cellular activity required by eukaryotes throughout evolution.

The algal PDI has many properties in common with previously isolated vertebrate PDIs (Hillson *et al.*, 1984; Freedman, 1989; Freedman *et al.*, 1989), i.e. dimeric form, subunit and dimeric M_r , optimal pH and temperature, and relative heat-stability. The proposed active site of vertebrate PDI (Hillson & Freedman, 1979) seems to be conserved through evolution, because it shows a high sequence identity with the active sites of thioredoxins isolated from bacteria, plants and animals (Edman *et al.*, 1985; Freedman *et al.*, 1988; Parkkonen *et al.*, 1988). The antibody data, however, indicate that other regions of the algal protein may show less identity with the vertebrate enzyme, because no cross-reactivity was detected between anti-(algal PDI) antibody and the vertebrate protein, or vice versa. Previously it was shown that polyclonal antibodies against vertebrate PDI cross-react with PDI from other vertebrate species (Koivu *et al.*, 1987) and, in the present study, antibodies against the bovine enzyme recognized chick PDI.

In vertebrates, PDI is associated with prolyl 4-hydroxylase, two molecules incorporating into the prolyl 4-hydroxylase tetramer (see Kivirikko *et al.*, 1989, 1990). Unlike the PDI in vertebrates, the *C. reinhardtii* PDI does not function as a component of the algal prolyl 4-hydroxylase. This was clearly demonstrated by the different binding properties of PDI and prolyl 4-hydroxylase to ion-exchange matrices and in their different elution positions on gel filtration (Kaska *et al.*, 1987, 1988). Furthermore, it has been shown that purified algal prolyl 4-hydroxylase does not contain any detectable PDI activity (Myllylä *et al.*, 1989).

At present, the role of PDI in the formation of disulphide bonds in specific secretory proteins of *C. reinhardtii* has not been determined. The cell-wall proteins of *C. reinhardtii* have been partially characterized, but their intramolecular disulphide bonds have not been investigated. The mobility of some wall components on SDS/PAGE, however, is increased by treatment with reducing agents (Monk, 1989), which implies the presence of intermolecular disulphide bonds. In wheat it has been shown that PDI catalyses the formation of disulphide linkages in storage proteins (Roden *et al.*, 1982).

In the present study PDI could be immobilized to an antibody-Protein A-Sepharose matrix in an active form. Disulphide-containing recombinant proteins cloned and expressed in bacteria are often produced in a scrambled and insoluble form, which limits their use (Mitraki & King, 1989). PDI has been shown to stimulate rearrangement of the disulphide bonds in many proteins and to improve their biological activity (Koivu & Myllylä, 1986; Bulleid & Freedman, 1988; Okumura *et al.*, 1988; Tang *et al.*, 1988). It would be worthwhile to test if the immobilized PDI, which can be easily removed by centrifugation, would enhance the activity of recombinant proteins with incorrect disulphide linkages.

To date, the cDNAs representing the mRNA encoding PDI have been sequenced from human (Pihlajaniemi *et al.*, 1987), rat (Edman *et al.*, 1985), mouse (Gong *et al.*, 1988), chick (Kao *et al.*, 1988) and bovine (Yamauchi *et al.*, 1987) tissues. The similarities of PDI among these species are high, ranging from 80 to 97% at the protein and DNA levels (see Kivirikko *et al.*, 1989, 1990). The algae, however, are evolutionarily distant from vertebrates, and screening of algal cDNA libraries with labelled vertebrate PDI cDNA probes yielded no positive clones. Furthermore, no hybridization could be detected under non-stringent conditions when similar probes were incubated with Northern and Southern blots containing *C. reinhardtii* polyadenylated RNA and DNA respectively. The highly specific antibodies prepared in the present study now provide a unique tool for screening a λ gt11 expression library of *C. reinhardtii* cDNA. The identification and

isolation of clones for the algal PDI will enable direct comparison of the nucleotide and amino acid sequences of the algal and vertebrate enzymes.

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REFERENCES

- Barbieri, L., Battelli, M. G. & Stirpe, F. (1982) Arch. Biochem. Biophys. **216**, 380–383
- Boado, R. J., Campbell, D. A. & Chopra, I. J. (1988) Biochem. Biophys. Res. Commun. **155**, 1297–1304
- Bradford, M. (1976) Anal. Biochem. **72**, 248–254
- Brockway, B. E. & Freedman, R. B. (1984) Biochem. J. **219**, 51–59
- Bulleid, N. J. & Freedman, R. B. (1988) Nature (London) **335**, 649–651
- Carmichael, D. F., Morin, J. E. & Dixon, J. E. (1977) J. Biol. Chem. **252**, 7163–7167
- Cheng, S.-Y., Gong, Q.-H., Parkison, C., Robinson, E. A., Apella, E., Merlino, G. T. & Pastan, I. (1987) J. Biol. Chem. **262**, 11221–11227
- Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A. & Rutter, W. J. (1985) Nature (London) **317**, 267–270
- Forster, S. J. & Freedman, R. B. (1984) Biosci. Rep. **2**, 223–229
- Freedman, R. B. (1989) Cell (Cambridge, Mass.) **57**, 1069–1072
- Freedman, R. B., Brockway, B. E. & Lambert, N. (1984) Biochem. Soc. Trans. **12**, 929–932
- Freedman, R. B., Hawkins, H. C., Murant, S. J. & Reid, L. (1988) Biochem. Soc. Trans. **16**, 96–99
- Freedman, R. B., Bulleid, N. J., Hawkins, H. C. & Paver, J. L. (1989) Biochem. Soc. Symp. **55**, 167–192
- Geetha-Habib, M., Noiva, R., Kaplan, H. A. & Lennarz, W. J. (1988) Cell (Cambridge, Mass.) **54**, 1053–1060
- Gong, Q., Fukuda, T., Parkison, C. & Cheng, S.-Y. (1988) Nucleic Acids Res. **16**, 1203
- Grynberg, A., Nicolas, J. & Drapron, R. (1978) Biochimie **60**, 547–551
- Hawkins, H. C. & Freedman, R. B. (1976) Biochem. J. **159**, 385–393
- Hillson, D. A. & Freedman, R. B. (1979) Biochem. Soc. Trans. **7**, 573–574
- Hillson, D. A. & Freedman, R. B. (1980) Biochem. J. **191**, 373–388
- Hillson, D. A., Lambert, N. & Freedman, R. B. (1984) Methods Enzymol. **107**, 281–294
- Ibbetson, A. L. & Freedman, R. B. (1976) Biochem. J. **159**, 377–384
- Kao, W. W.-Y., Nakazawa, M., Aida, T., Everson, W. V., Kao, C. W.-C., Seyer, J. M. & Hughes, S. H. (1988) Connect. Tissue Res. **18**, 157–174
- Kaska, D. K., Günzler, V., Kivirikko, K. I. & Myllylä, R. (1987) Biochem. J. **241**, 483–490
- Kaska, D. D., Myllylä, R., Günzler, V., Gibor, A. & Kivirikko, K. I. (1988) Biochem. J. **256**, 257–263
- Kivirikko, K. I., Myllylä, R. & Pihlajaniemi, T. (1989) FASEB J. **3**, 1609–1617
- Kivirikko, K. I., Helaakoski, T., Tasanen, K., Vuori, K., Myllylä, R., Parkkonen, T. & Pihlajaniemi, T. (1990) Ann. N.Y. Acad. Sci., in the press
- Kivirikko, K. I., Helaakoski, T., Tasanen, K., Vuori, K., Myllylä, R. & Pihlajaniemi, T. (1990) Ann. N.Y. Acad. Sci., in the press
- Koivu, J. & Myllylä, R. (1986) Biochemistry **25**, 5982–5986
- Koivu, J. & Myllylä, R. (1987) J. Biol. Chem. **262**, 6159–6164
- Koivu, J., Myllylä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K. & Kivirikko, K. I. (1987) J. Biol. Chem. **262**, 6447–6449
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Lambert, N. & Freedman, R. B. (1983) Biochem. J. **213**, 225–234
- Mitraki, A. & King, J. (1989) Biotechnology **7**, 690–697
- Monk, B. C. (1988) Planta **176**, 441–450
- Munro, S. & Pelham, H. R. B. (1987) Cell (Cambridge, Mass.) **48**, 899–907
- Myllylä, R. & Oikarinen, J. (1983) J. Biochem. Biophys. Methods **7**, 115–121
- Myllylä, R., Kaska, D. D. & Kivirikko, K. I. (1989) Biochem. J. **263**, 609–611
- Okumura, K., Miyake, Y., Wakayama, H., Miyake, T., Murayama, K., Seta, K., Taguchi, H. & Shemabayashi, Y. (1988) Agric. Biol. Chem. **52**, 1735–1739
- Parkkonen, T., Kivirikko, K. I. & Pihlajaniemi, T. (1988) Biochem. J. **256**, 1005–1011

- Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M.-L., Koivu, J. & Kivirikko, K. I. (1987) *EMBO J.* **6**, 643–649
- Roden, L. T., Mifflin, B. J. & Freedman, R. B. (1982) *FEBS Lett.* **138**, 121–124
- Solter, K. M. & Gibor, A. (1977) *Plant Sci. Lett.* **8**, 227–231
- Steiner, R. F., De Lorenzo, F. & Anfinsen, C. B. (1965) *J. Biol. Chem.* **240**, 4648–4651
- Tang, J.-G., Wang, C.-C. & Tsou, C.-L. (1988) *Biochem. J.* **255**, 451–455
- Towbin, H., Staehelin, T. & Cordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Yamauchi, K., Yamamoto, T., Hayashi, H., Koya, S., Takikawa, H., Toyoshima, K. & Horiuchi, R. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1485–1492

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