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Purification, properties and influence of dietary copper on accumulation and functional activity of lysyl oxidase in rat skin

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Lysyl oxidase (protein-lysine 6-oxidase; EC 1.4.3.13) is a copper-containing enzyme that functions extracellularly and catalyses the oxidative deamination of peptidyl lysine. Lysyl oxidase was purified 150–175-fold from urea extracts of rat skin and uteri. Features of the enzyme were similar to those reported previously for lysyl oxidase obtained from rat aorta and bovine ligamenture. However, both ~ 40 and ~ 32 kDa polypeptide chains could be isolated from rat skin with apparent lysyl oxidase activity. Antibodies raised in chickens against the ~ 40 kDa form of lysyl oxidase detected the ~ 32 kDa form in immunoblots. Consequently it is inferred that the ~ 32 kDa form of lysyl oxidase is processed from the ~ 40 kDa form of the enzyme. The antibodies were also used to prepare anti(rat lysyl oxidase) affinity columns to facilitate the separation of lysyl oxidase from other proteins in studies to assess the extent to which lysyl oxidase serves as a reservoir for skin copper. At 16 h after an oral dose of copper, as ^{67}Cu, about 6–8 % of the total ^{67}Cu incorporated into rat skin was found in association with lysyl oxidase. The lysyl oxidase concentration in rat skin was 2.5–7.5 nmol/g (determined by e.l.i.s.a.). Changing the copper status of rats by feeding a diet deficient in copper did not appear to influence lysyl oxidase accumulation in skin nor the percentage of incorporation of ^{67}Cu in skin as lysyl oxidase. However, when rats were deprived of copper, the functional activity of lysyl oxidase in skin was one-third to one-half the normal values.

INTRODUCTION

Lysyl oxidase (protein-lysine 6-oxidase; EC 1.4.3.13) is an important enzyme in the post-translational modification of collagen and elastin (Rucker & Dubick, 1984; Tinker & Rucker, 1985; Kagan, 1986; Ricard-Blum & Ville, 1989). Lysyl oxidase catalyses extracellularly the oxidative deamination of specific lysine and hydroxylysine residue to peptidyl-aldehydes, such as α-aminoadipic-δ-semialdehyde. The aldehydic functions then serve as precursors to elastin and collagen cross-linking amino acids.

Lysyl oxidase has been isolated and partially characterized from human (Kuivaniemi *et al.*, 1984), avian (Narayanan *et al.*, 1974; Stassen, 1976) and bovine sources (Cronlund & Kagan, 1986; Williamson & Kagan, 1986). Lysyl oxidase from bovine sources exists as four isoforms with molecular masses of about 32 kDa and contains little, if any, carbohydrate (Cronlund & Kagan, 1986; Williamson & Kagan, 1986). Lysyl oxidase may serve a structural role in some extracellular matrices (Kagan *et al.* 1986). Trackman *et al.* (1990) have recently cloned rat aorta lysyl oxidase. Lysyl oxidase is translated from a 2.7 kb mRNA of which 1.16 kb represent non-coding sequences. Trackman *et al.* (1990) and Wakasaki & Ooshima (1990) have also demonstrated, in cell-free translation assays, that the initial product of lysyl oxidase mRNA translation has a molecular mass of 46–48 kDa.

The functional activity of lysyl oxidase is influenced by hormones (Bronson *et al.*, 1987), factors that influence collagen and elastin conformation (Gabriel & Kagan, 1988; Tang *et al.*, 1989) and nutritional factors (Harris, 1976; Rayton & Harris, 1979; Harris & DiSilvestro, 1981; Opsahl *et al.*, 1982; Dubick *et al.*, 1985). As the metal cofactor for lysyl oxidase, copper is particularly important (Tinker & Rucker, 1985). Functional activity has been shown to vary as much as 10-fold in response to varying dietary copper (Harris, 1976; Rayton & Harris, 1979; Opsahl *et al.*, 1982). A dietary deficiency of pyrroloquinoline quinone has been shown to depress the accumulation of lysyl oxidase in mouse skin (Kilgore *et al.*, 1989). Clearly a quinone, possibly 6-hydroxydopa (3,4,6-trihydroxyphenylalanine), serves at the catalytic centre of lysyl oxidase (Gacheru *et al.*, 1989; Janes *et al.*, 1990). Lysyl oxidase is also inhibited by vicinal diamines (Gacheru *et al.*, 1989), heparin (Gabriel & Kagan, 1988), short-chained aminonitrites and semicarbazides, hydrazines and some alkylating agents (Kagan, 1986).

We have purified lysyl oxidase from rat uteri and skin by methods that were adapted from Kagan and his associates (Cronlund & Kagan, 1986; Williamson & Kagan, 1986), but with modification. The product of purification was used as an antigen for antibody production. The antibody was then used to determine tissue concentrations of lysyl oxidase by e.l.i.s.a. and in the preparation of lysyl oxidase affinity columns to facilitate the separation of lysyl oxidase from other proteins. The incorporation of copper into lysyl oxidase was then studied in rats rendered either copper-sufficient or -deficient by varying dietary copper intake. Moreover, it could also be inferred that lysyl oxidase undergoes proteolytic processing, i.e. the ~ 32 kDa form of lysyl oxidase appears to evolve from a ~ 40 kDa precursor.

EXPERIMENTAL

Materials

New England Nuclear Corp. (Boston, MA, U.S.A.) was the source of L-[4,5-^{3}H]lysine (sp. radioactivity 83 Ci/mmol). ^{67}Cu (as $CuSO_4$) was prepared at the Brookhaven National Laboratories (Upton, Long Island, NY, U.S.A.) and was 2.8 mCi/μg (half-life 61.88 h). At the time of use this product contained ^{61}Cu (half-life 3.2 h) and ^{64}Cu (half-life 12.7 h) as impurities, but at levels of less than 5 %. Medium 199 devoid of lysine with Hanks salts was

Abbreviation used: TETA, triethylenetetra-amine.

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purchased from Gibco (Grand Island, NY, U.S.A.). Reagents and supplies for antibody preparations were obtained from Antibodies Inc. (Woodland, CA, U.S.A.) and RIBI Immunochemical Research (Hamilton, MO, U.S.A.). Electrophoretic and column-chromatographic supplies were products of Bio-Rad (Richmond, CA, U.S.A.), Pharmacia (Piscataway, NJ, U.S.A.), or Millipore (Bedford, MA, U.S.A.). Actigel-Ald was obtained from Sterogene (Arcadia, CA, U.S.A.), and nitrocellulose from Schleicher and Schuell (New York, NY, U.S.A.). All other chemicals were of the highest quality available from Fisher Scientific (Pittsburgh, PA, U.S.A.), Pierce Chemical Co. (Rockford, IL, U.S.A.), Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) or Sigma Biochemical Co. (St. Louis, MO, U.S.A.).

Insoluble elastin for lysyl oxidase affinity columns was prepared from the aortae of chicks fed a copper-deficient diet [see Rucker (1982) for a description of materials] from the day of hatching for 21 days. Elastin was isolated from aortas by hot-alkali extraction (0.1 M-NaOH; 90 °C for 45 min).

Assay for lysyl oxidase

Lysyl oxidase was assayed by a modification of the ^{3}H-release assay of Pinnell & Martin (1968), for which substrate was prepared using aortae from 7-day-old chicks. Briefly, the aortae were first minced (1 mm^3); 2–3 g of tissue were next incubated in 25 ml of medium 199 containing L-[4,5-^{3}H]lysine (50 μCi/ml) and β-aminopropionitrile, a lysyl oxidase inhibitor (50 μg/ml). The incubations were for 12 h under an O_2/CO_2 (19:1) atmosphere. The tissue was homogenized in phosphate-buffered (0.05 M, pH 7.4) saline containing 50 mM-L-lysine, and washed until soluble radioactivity as L-[4,5-^{3}H]lysine was negligible. As a final wash step, the substrate was suspended in 0.01 M-HCl, collected by centrifugation and freeze-dried for storage. About 1 μCi of ^{3}H was incorporated/10 mg of minced aortae.

In typical assays, 150000–200000 c.p.m. of the ^{3}H-labelled aortic substrate was used. Enzyme fractions were added to substrate suspended in 0.1 M-sodium borate buffer, pH at 8.0, containing 0.15 M-NaCl. Samples were incubated at 45 ° for 2 h (assay volume 1.5 ml). Dialysed crude extracts of skin or uterus (about 100–200 μg, as protein) usually caused a release of 1000–1500 c.p.m. of ^{3}H. The assays were performed with substrate alone, with enzyme or with enzyme preincubated (60 min) with 50 μg of β-aminopropionitrile/ml. Urea in the assayed fractions was also adjusted by dilution or dialysis (against 0.1 M-sodium borate buffer, pH 8.0) so that the concentration was never higher than 0.2 M-urea in a given assay.

The reactions were stopped by the addition of 0.2 ml of 50% (w/v) trichloroacetic acid. Released ^{3}H was determined after centrifugation and passage of each sample over a 0.5 cm × 3.0 cm column of AG 50W-X8 (H^+) resin (Myers *et al.*, 1985). After a wash with 2 column vol. of distilled water, the eluate was counted for radioactivity by liquid-scintillation spectrometry.

Purification of lysyl oxidase

Uteri (days 18 and 19 of pregnancy) or skin from 12–14-week-old Sprague–Dawley rats were used as starting materials. When skin was the source, the tissue (400–500 g) was shaved, coarsely minced, frozen in liquid N_2 and pulverized to a fine powder in a commercial Waring blender. Similarly, uteri were also coarsely minced, but were homogenized directly into phosphate-buffered saline. After pulverization or homogenization the tissue was next stirred into phosphate-buffered saline (4:1, v/w) and washed twice (1 h each wash) to remove lysyl oxidase inhibitors and readily solubilized protein (Narayanan *et al.*, 1982).

The washed pellet (15000 *g*, 30 min) was next extracted (5:2, v/w) for 24 h at 4 °C with buffered (0.1 M sodium borate, pH 8.0) 6 M-urea. The urea solution was passed through a mixed-bed resin (AG 501-X8) before use. The pellet was suspended in the urea solution. After centrifugation, the supernatant fractions from the extractions were combined and filtered.

The filtrate was then adjusted to 2 M-urea, and solid $(NH_4)_2SO_4$ was added slowly to 75% saturation. Proteins were allowed to aggregate for 12–14 h and pelleted (10000 *g*, 30 min). The pelleted protein was next redissolved in 2 M-urea/0.1 M-sodium borate, pH 8.0, to about 10–20 mg/ml and dialysed against the same solution. Insoluble elastin from copper-deficient chick aorta (see under 'Materials' above) was then added to the dialysed samples (1 g of elastin/g of dissolved protein). This mixture was stirred (3–4 h), poured into a column (6 cm diameter), and extensively washed with 4 column vol. of 2 M-urea, followed by 4 column vol. of phosphate-buffered saline. The bulk of the lysyl oxidase activity, 60–70% of that applied, could then be eluted by a 2 column vol. addition of 6 M-urea buffered with 0.1 M-sodium borate buffer.

The final step in purification was gel-exclusion chromatography on a column (2.5 cm × 60 cm) of Sephacryl S-200 using 6 M-urea/0.1 M-sodium borate, pH 8.0, as eluent. On occasions, a small portion (5–10%) of the protein that was applied was allowed to react with β-amino[^{14}C]propionitrile as described by Tang *et al.* (1983). The rationale was based on the specificity of β-aminopropionitrile for lysyl oxidase as a covalently bound inhibitor. Radioactivity associated with the enzyme provided an additional index for monitoring lysyl oxidase eluted from the gel-permeation columns. Protein fractions containing functional activity were electrophoresed as described by Laemmli (1970). Protein was detected by staining a portion of the gel with either Coomassie Brilliant Blue R250 or silver (Heng-Khoo *et al.*, 1979; Romero *et al.*, 1986; Tinker *et al.*, 1986, 1990).

Lysyl oxidase compositional studies and antibody production

Samples of enzyme after gel permeation were subjected to preparative PAGE for antibody production and composition studies. Protein (50–100 μg) was applied to a 6–7 cm trough of acrylamide and electrophoresed (Laemmli, 1970). Lanes of protein corresponding to lysyl oxidase were then eluted on to sheets of nitrocellulose or Immobylon P (Millipore Corp., Bedford, MA, U.S.A.). The enzyme bound to Immobylon P was used for compositional studies. Amino acid analysis was performed after acid hydrolysis (6 M-HCl for 36 h at 110 ° in sealed tubes), and attempts were made to obtain *N*-terminal-amino-acid-sequence information (cf. Heng-Khoo *et al.*, 1979; Trackman *et al.*, 1990).

For antibody production the protocols outlined by Bade & Stegemann (1984) and Carroll & Stollar (1983) were followed. Briefly, hens were injected intraperitoneally with 20–30 μg of enzyme (bound to nitrocellulose) at multiple sites. The birds received biweekly booster injections with 10–20 μg of enzyme. Adequate serum titres were observed after two or three booster injections. The antibody titres for hen serum were 1:2000 as estimated by e.l.i.s.a. After 4 weeks, eggs were also collected for the extraction and isolation of anti-(lysyl oxidase) antibodies. The titres for egg yolks were about 1:500. To isolate immunoglobulins from eggs, yolks were collected and lipid was removed by extraction into propane-2-ol (−20 °C). After three washes with propan-2-ol, the residue was next washed twice with acetone (−20 °C). This residue was dried and extracted with 0.01 M-NaCl and 0.01% NaN_3. Further purification of egg yolk was achieved by DEAE-cellulose chromatography (Bade & Stegemann, 1984; Carroll & Stollar, 1983). The serum antibodies were used directly or after precipitation of the IgG fraction with $(NH_4)_2SO_4$.

An indirect method for e.l.i.s.a. of lysyl oxidase was adopted from that described by Voller *et al.* (1980). Goat anti-chick

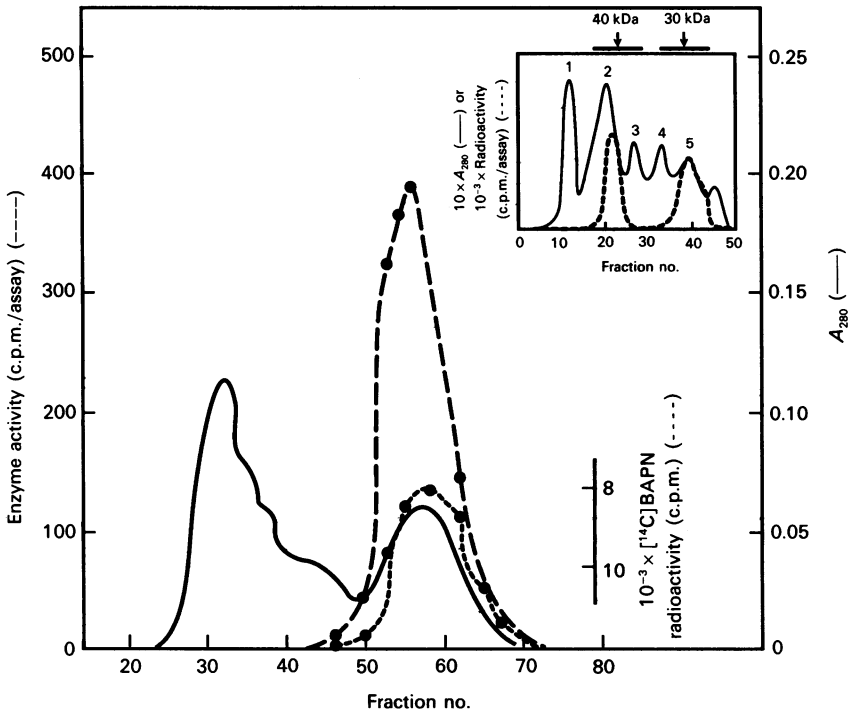

Fig. 1. Elution of rat uterus lysyl oxidase activity from a column of Sephacryl S-200 (2.5 cm × 60 cm)

About 10 mg of protein, which was obtained by elution from an elastin affinity column (see the text), was applied. Chromatography was performed by using 0.1 M-sodium borate buffer, pH 8.0, containing 6 M-urea, as eluent. Functional activity and radioactivity as β-amino[^{14}C]propionitrile ([^{14}C]BAPN) was eluted as a broad peak corresponding to 30–35 kDa proteins. The inset depicts the elution of rat skin lysyl oxidase activity from a column of Sephacryl S-200 (2.5 cm × 45 cm) but after only the urea-extraction step and immediate concentration by $(NH_4)_2SO_4$ fractionation. Functional activity, assayed after dialysis, was observed to be eluted as 40 (±5) or 30 (±5) kDa polypeptide chains.

antiserum conjugated to horseradish peroxidase was used for detection. The substrate solutions contained 20 mg of 3,3′,5,5′-tetramethylbenzidine and 3 μl of 30% H_2O_2 in 20 ml of 0.1 M-sodium acetate/citric acid buffer, pH 6.0. Specificity was assessed in Western blots (Tinker *et al.*, 1990). Lysyl oxidase was transferred to nitrocellulose and detected by using hen anti-(lysyl oxidase) serum at 1:2000 dilution. The nitrocellulose was blocked with a 3% (w/v) BSA/phosphate-buffered saline solution. Goat anti-chicken immunoglobulin conjugated to horseradish peroxidase was used for detection (Voller *et al.*, 1980).

Incorporation of ^{67}Cu into lysyl oxidase

Groups of female rats (200–210 g) fed diets sufficient (12 μg/g) or deficient (< 0.5 μg/g) in dietary Cu were used to assess ^{67}Cu incorporation into skin and associated proteins. The diet composition and general protocols were those described by Chung *et al.* (1988). The rats in the Cu-deficient group were given a diet containing the Cu chelator triethylenetetra-amine (TETA) at 1% (w/w) of the diet for 1 week. Then the Cu-deficient diet without added TETA was fed for the remaining 4 weeks to the Cu-deficient group. On the day before the isotope gavage, rats from both diet groups were given the Cu-deficient diet at 16:00 h. At 12:00 h food cups were removed from all rats. Rats were gavaged at 6:00 h with a dose of 28 μCi of ^{67}Cu in 0.5 ml of a 25% slurry of the Cu-deficient diet followed by 0.5 ml of saline. All rats were given the Cu-deficient diet after gavage. At 10:00 h the respective Cu-supplemented and Cu-deficient rats were returned to the cages and both groups were fed *ad libitum*. The incorporation of ^{67}Cu into skin and other tissues was then estimated at 8, 16, 24, 32, 64 or 128 h. The 16 h samples were used to assess ^{67}Cu incorporation into lysyl oxidase.

First, samples of 1 g or more of skin were counted for radioactivity in a γ-radiation counter with the windows set from 10 to 500 keV. The samples were next homogenized into phosphate-buffered saline (1:4, w/v), and the tissue was re-extracted using 6 M-urea buffered with 0.1 M-sodium borate, pH 8.0. The urea extract was dialysed until no radioactivity was detected in the dialysis residue. The dialysis residue was then passed over a column of Actigel-ald to which anti-(lysyl oxidase) antibody was ligated. The ligation procedure was that outlined by the supplier (Sterogene, Arcadia, CA, U.S.A.). The column capacities were determined in preliminary experiments by estimating bound and unbound functional activity. For the assays, affinity columns were first equilibrated with 0.1 M-borate buffer, and lysyl oxidase-containing samples were loaded on to the columns in the same buffer. The columns were next washed with 2 column vol. of 0.1 M-sodium borate buffer, pH 8.0, 2 column vol. of 0.5 M-NaCl/0.1 M-borate buffer and 2 column vol. of the 0.1 M-borate buffer. Lysyl oxidase was released upon elution with 4 M-urea in 0.1 M-borate buffer.

Other assays

Tissue Cu concentrations were measured by flame-atomic-absorption spectrophotometry after wet ashing with HNO_3 as described by Clegg *et al.* (1981). Data were analysed statistically by Student's *t* test.

RESULTS

Purification of lysyl oxidase

Fig. 1 shows a typical elution profile of lysyl oxidase and associated proteins after chromatography on columns of Sephacryl S-200. The elution pattern was similar for fractions from either rat skin or uterus.

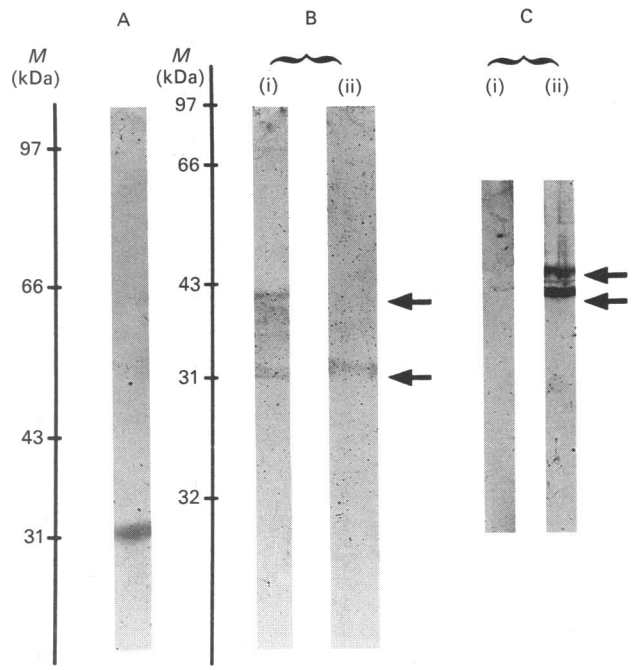

Fig. 2. SDS/PAGE of lysyl oxidase-containing fractions obtained after Sephacryl S-200 chromatography or absorption to, and elution from, anti-(lysyl oxidase) affinity columns (cf. the text)

A, purified rat skin lysyl oxidase was obtained after elution from an anti-(lysyl oxidase) affinity column and stained with Coomassie Blue. For this preparation, only the 32 kDa form is observed. About 10 μg of protein were applied to a 5%-(w/v) polyacrylamide gel. B, fractions of rat skin lysyl oxidase after Sephacryl S-200 chromatography and electrophoresis in 7.5% polyacrylamide gels (stained with silver). Note the diffuse bands at $\sim$ 40 kDa and $\sim$ 32 kDa (i). With storage, often only the 32 kDa form was observed (ii), as in A. C, immunoblot of a fresh urea extract of skin using antibody against the 40 kDa form of lysyl oxidase for detection. When fresh extracts are examined (ii), bands at both 40 and 30 kDa are often observed (electrophoreses in 12% polyacrylamide). Carbonic anhydrase, ovalbumin, BSA and muscle phosphorylase were used as molecular-mass (M) markers. The immunoblot to the left (i) is for preimmune serum.

To arrive at this step, 10–12 mg of crude protein were usually extracted into buffered 6 M-urea per gram of skin or uterus, i.e. 4–5 g of protein or $(4–8) \times 10^7$ units of activity from the total tissue sample (400–500 g). Next, a 50–80-fold purification was usually achieved by allowing lysyl oxidase to be absorbed on to insoluble, but partially cross-linked, elastin, i.e. a recovery of $(2–4) \times 10^7$ units of activity associated with 40–60 mg of protein. Fractions containing 5×10^5 to $(1–2) \times 10^6$ units of activity/mg could be obtained at the Sephacryl S-200 gel-exclusion step.

When we obtained values for specific activity that exceeded 10^6 units/mg, such values were taken to indicate a very high degree of purity, on the basis of the observations by Cronlund & Kagan (1986). They estimated that, against chick-embryo aorta substrate, the theoretical yield of lysyl oxidase functional activity is about 4×10^6 units/mg of purified enzyme, where one unit is defined as 1 d.p.m. of ^{3}H released in 2 h.

The fold purification was 150–175 after gel-permeation chromatography relative to the specific activity of the initial urea extracts, or about 2000–3000-fold relative to the total protein content of skin or uterus. Activity could be monitored by assaying functional activity or monitoring protein to which β-amino[^{14}C]propionitrile was covalently associated. In the absence of urea the rat enzyme polymerized in a manner similar to that described by Burbelo *et al.* (1985). When stored at -20 °C for more than 4–5 weeks, losses in activity were often greater than 50%.

Data for the amino acid composition of the enzyme bound to Immobylon P were in keeping with those obtained previously for lysyl oxidase (Kagan, 1986). Attempts to establish an *N*-terminal sequence were not successful, suggesting that the *N*-terminus of lysyl oxidase is blocked (also cf. Trackman *et al.*, 1990).

Antibody specificity and evidence for proteolytic processing

For given preparations, gel-permeation chromatography often resulted in two peaks of activity (inset to Fig. 1). When fractions containing activity were assessed by electrophoresis in SDS/polyacrylamide gels, bands at $\sim$ 32 and/or $\sim$ 40 kDa were observed (Fig. 2). Moreover, antibodies raised against the $\sim$ 40 kDa form of lysyl oxidase reacted in immunoblots with the $\sim$ 32 kDa form of the protein. It was noteworthy that, in fresh-tissue extracts, the $\sim$ 30 kDa form of lysyl oxidase was observed (Figs. 1 and 2). We inferred from the observations that the $\sim$ 32 kDa form of lysyl oxidase was derived from a $\sim$ 40 kDa form.

With respect to chicken serum antibodies, the ability of the antibody to absorb lysyl oxidase functional activity (Fig. 3) and behaviour in immunoblots were used as evidence for specificity. When lysyl oxidase antibody was bound to the wells of e.l.i.s.a. plates and further blocked with BSA, the addition of highly purified and functionally active lysyl oxidase resulted in absorption of enzymic activity by the bound antibody that was directly related to antibody concentration (Fig. 3).

Quantification of lysyl oxidase

In e.l.i.s.a., estimates of lysyl oxidase in rat skin ranged from 100 to 200 μg/g of skin. These values are in keeping with assessments from functional assays, assuming that 1 mg of lysyl oxidase corresponded to the release of about 10^6 units of lysyl oxidase. The values are also similar to those reported previously by Burbelo *et al.* (1985) on the lysyl oxidase content of bovine aorta and by Schackleton & Hulmes (1990) on the lysyl oxidase content of piglet skin. The functional activity of lysyl oxidase ranged from 160000 to 190000 units/g of skin from the Cu-supplemented rats compared with 500000–590000 units/g of skin from Cu-deficient rats ($P < 0.01$ for n = six to eight samples from individual rats). Furthermore, we could detect no difference in the amounts of lysyl oxidase as protein, i.e., in e.l.i.s.a., in the skin of rats when samples from Cu-deficient and -sufficient rats were compared.

Assessment of Cu status

As expected, plasma and liver Cu concentrations were reduced with Cu deficiency (Table 1). However, there was no change in the concentration of Cu in skin between the groups. Further, the rat maintains the concentration of Cu in skin at levels similar to those in liver (cf. the Discussion section). Data for the incorporation and disappearance of ^{67}Cu in rat skin are given in Fig. 4. These data suggest two or three major compartments for skin Cu with an apparent turnover in supplemented rats of 10–20 h for the more rapidly mobilized pool(s) and several days for the more slowly mobilized pools.

Incorporation of ^{67}Cu into lysyl oxidase

About 5% of the oral dose of ^{67}Cu was deposited into skin within 8 h (cf. Fig. 4) [assuming the proportion of skin to total body weight is about 1:6 (Owens, 1964)]. AT 16 h, 6% of the total ^{67}Cu in skin from Cu-supplemented rats (3.2×10^4 c.p.m./g)

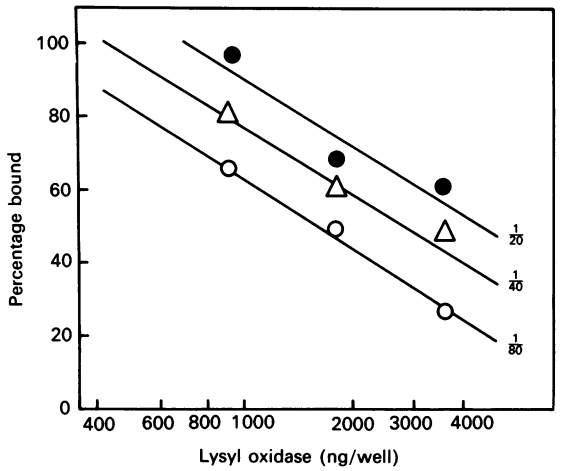

Fig. 3. Binding of lysyl oxidase to antibodies in chicken serum

From the lot of serum used in the present study, a 1/20 dilution of antiserum coated on to the well of an e.l.i.s.a. titre plate bound about 0.7–0.9 μg of lysyl oxidase. Wells were coated with three different dilutions of antiserum (1/20, 1/40 and 1/80). Differing quantities of lysyl oxidase containing known amounts of protein and activity were then added to each well. After a 2 h incubation, unbound enzymic activity was determined to estimate the percentage of bound lysyl oxidase.

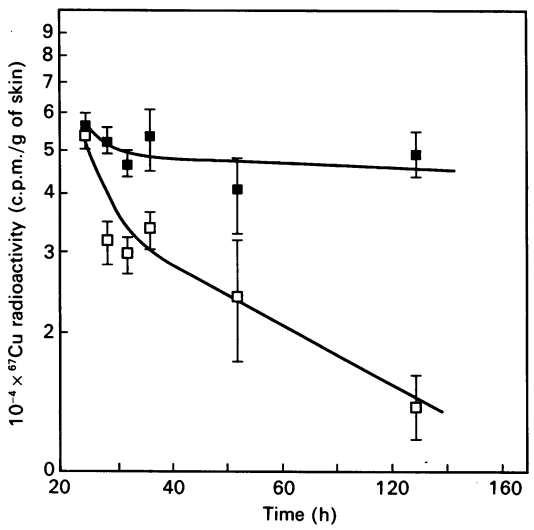

Fig. 4. Incorporation of ^{67}Cu into (and retention in) skin from Cu-supplemented (□) and -deficient (■) female rats (200–210 g) after an oral dose of ^{67}Cu

The diet protocol is described in the text. Rats were gavaged with a dose of 28 μCi of ^{67}Cu in an 0.5 ml slurry of Cu-deficient diet in water (1:3, w/v), followed by 0.5 ml of saline.

Table 1. Effect of dietary Cu on the Cu concentration in selected tissues

The deficient diet contained less than 0.5 μg of Cu/g. Cu was added to the supplemented diet at 12 μg/g. Values are means ± S.E.M. ($n = 8$). An asterisk indicates significance at $P < 0.001$.

		Cu concn.		
	Units ...	(nmol/ml)	(nmol/g)	
Diet	Tissue ...	Plasma	Liver	Dorsal skin
Supplemented		16.7 ± 0.4	81.5 ± 5.4	41.6 ± 9.1
Deficient		8.0 ± 1.8*	50.1 ± 3.3*	53.8 ± 12.2

was associated with lysyl oxidase (1.8×10^3 c.p.m.). Of the 5.3×10^4 c.p.m./g as ^{67}Cu in the skin of Cu-deficient rats, 4.4×10^3, or 8%, of the total ^{67}Cu was associated with lysyl oxidase. Normal rat skin contains about 40 nmol of copper and from 2.5 to 7.5 nmol of lysyl oxidase/g. Consequently, the same ratio of Cu in lysyl oxidase to total skin Cu is also obtained by direct analysis. Differences in the incorporation of Cu into skin or lysyl oxidase when Cu-deficient and supplemented rats were compared (16 h post-intubation) were assumed to be differences resulting from the lower plasma Cu values for deficient rats or differences in apparent turnover (cf. Table 1 and Fig. 4).

DISCUSSION

The availability of insoluble elastin from Cu-deprived chicks facilitated the rapid isolation of two forms of lysyl oxidase. After the production of an anti-(lysyl oxidase) antiserum, it was also possible to examine various features of lysyl oxidase metabolism *in vivo*, particularly in response to varying the availability of Cu, an important lysyl oxidase cofactor (Kagan, 1986).

With respect to lysyl oxidase and Cu, Balthrop *et al.* (1982), Harris (1976) and Rayton & Harris (1979) compared and examined previously putative binding proteins in liver and aorta. In liver, Cu is associated initially (< 6 h) with low-molecular-mass protein (< 10 kDa), possibly metallothionein, whereas in connective tissue, such as aorta, only trace amounts of Cu are associated with low-molecular-mass proteins. Measurable amounts of isotope in aorta, however, were incorporated into protein fractions with the characteristics of superoxide dismutase and lysyl oxidase. Harris (1976) demonstrated that the uptake of Cu into aorta leads to a rapid activation of lysyl oxidase. Rayton & Harris (1979) also showed that the incorporation of Cu into chick aorta lysyl oxidase is inhibited by cycloheximide but not by actinomycin D.

Likewise, for lysyl oxidase in rat skin, it may be inferred that Cu equilibrates rapidly with the active pool of enzyme. In order to maintain lysyl oxidase activity that corresponds to 3–6 nmol of enzyme/g of skin, 0.15–0.30 μg of Cu are theoretically needed for activity, which corresponds to 6–8% of the total Cu content of skin. That 6–8% of ^{67}Cu was associated with lysyl oxidase indicates relatively complete equilibration of copper into lysyl oxidase by 16 h. It also appears that functional activity of the enzyme is perturbed more by changes in plasma Cu levels or the Cu concentration of the vehicle that delivers Cu to skin than the levels of Cu in skin *per se*. Skin Cu levels did not change in response to Cu deprivation. Why this phenomenon occurs in skin, but not other tissues, is not known.

Our observations complement those by Trackman *et al.* (1990), who have characterized lysyl oxidase derived from a rat aorta cDNA library cloned into λgt_{11}. Their observations suggest that lysyl oxidase is synthesized first as a 48 kDa polypeptide chain with obvious tryptic-like cleavage sites that could yield products of ~32 and ~40 kDa. Moreover, it is noteworthy that, in a recent study by Wakasaki & Ooshima (1990), a 48 kDa form of lysyl oxidase was observed after cell-free translation. Although not emphasized by these authors, ~40 and ~32 kDa polypeptide chains were observed to precipitate with anti(lysyl oxidase) antibody. In previous studies, lysyl oxidase has been isolated, usually in its 32 kDa form, from relatively elastin-rich or cartilaginous sources, whereas a rela-

tively type I collagen-rich source, skin or uterus, was used here. One may speculate that specific proteolysis of the enzyme aids in directing specificity (collagen versus elastin) or specific features related to its matrix deposition.

With the information provided here and by Kagan (1986) on the limits of catalytic cycling by lysyl oxidase, it is also possible to speculate on the extent to which lysyl oxidase is rate-limiting in skin for connective-tissue protein cross-linking. For example, by assuming that lysyl oxidase efficiently catalyses 400 catalytic cycles/mol of enzyme, and knowing that rat skin contains 3–6 nmol of lysyl oxidase/g, it is possible to conclude that skin is relatively saturated with enzyme. The ability to generate at any given time 1.2–2.4 μmol of peptidyl α-aminoadipic-δ-semialdehyde in collagen and elastin as a cross-linking-amino-acid precursor is more than sufficient for the daily deposition of new elastin and collagen in skin. Much of the collagen and the elastin in skin turns over slowly [cf. Tinker & Rucker (1985) and references cited therein]. Consequently, the slow turnover and any deposition of new collagen/g of skin on a daily basis should be accommodated by the generation of only nanomolar amounts of lysine-derived cross-linking amino acids (Reiser *et al.*, 1987). In keeping with this impression, we have recently tried to demonstrate that collagen is abnormally cross-linked in skin from the Cu-deficient rat. However, collagen solubility and cross-linking were not particularly affected in Cu-deficient rats (J. Lee, N. Romero-Chapman & R. Rucker, unpublished work). The high content of lysyl oxidase in skin helps to explain this observation. It may also be proposed that a high concentration of enzyme would be required if the enzyme is immobilized as it becomes a part of the extracellular matrix (Kagan *et al.*, 1986).

As a final point, models that involve cofactor deprivation or perturbation can be useful in examining the novel strategies that are imposed to control the functional activities of extracellular enzymes and processes. It has been shown previously that, in Cu-depleted animals, repletion with Cu rapidly restores enzyme functional activity. This process requires the cellular uptake of Cu and active cellular processing (Harris, 1976; Rayton & Harris, 1979; Opsahl *et al.*, 1982). We can infer from our estimates of net accumulation and ^{67}Cu incorporation into lysyl oxidase in Cu-supplemented and depleted animals that the enzyme may be subject to daily turnover. Obviously, considerable information is still needed, however, to assess fully how lysyl oxidase is regulated and processed; this is an interesting problem, since the enzyme functions outside the cell on substrates with markedly different physical and chemical properties.

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