all complexes is given by eq 5.

$$E_T/v = \left[(k_5' + k_6 + k_{19} + k_{21})\frac{[NH_4OAc]}{K_7} + k_5' + k_{11} + k_9 + k_5'\left(k_{11} + k_{19}\frac{[NH_4OAc]}{K_7} \right)B \right] \Big/ \left[k_5'\left[(k_{19} + k_{21})\frac{[NH_4OAc]}{K_7} + k_9 + k_{11} \right] \right] \quad (5)$$

where

$$B = \frac{1 + \frac{[G]}{K_{13}} + \frac{[NH_4OAc]}{K_{22}}}{k_{15} + k_{17}\frac{[G]}{K_{13}} + k_{23}\frac{[NH_4OAc]}{K_{22}}} \quad (6)$$

and

$$(k_5')^{-1} = \phi_0 + \frac{\phi_1}{[O]} + \frac{\phi_2}{[G]} + \frac{\phi_{12}}{[O][G]} \quad (7)$$

is the reciprocal specific initial velocity of the burst phase in the absence of ammonium acetate (Colen et al., 1972; Brown et al., 1978). Assuming that only ERK and ERKN complexes make a substantial contribution to the absorbance at the end of the burst phase, the burst amplitude is given by

$$\frac{\Delta A_B}{el} = \frac{[NH_4OAc] + K_7}{K_7}[ERK] \quad (8)$$

where

$$[ERK] = k_5'E_T/\left[(k_5' + k_6 + k_{19} + k_{21})\frac{[NH_4OAc]}{K_7} + k_5' + k_{11} + k_9 + k_5'\left(k_{11} + k_{19}\frac{[NH_4OAc]}{K_7} \right)B \right] \quad (9)$$

Combining eq 5–9 yields eq 2 in the text.

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Purification and Enzymatic Properties of Lysyl Hydroxylase from Fetal Porcine Skin†

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ABSTRACT: Lysyl hydroxylase from fetal porcine skin is shown to bind in a highly specific manner to aminoethyl-Sepharose 4B. When coupled to ammonium sulfate fractionation and DEAE-cellulose chromatography, chromatography of lysyl hydroxylase preparations on aminoethyl-Sepharose 4B has yielded a highly purified (>95%) preparation of lysyl hydroxylase. The enzyme consists of two subunits with molecular weights of 70 000 and 115 000. The overall recovery of activity was 2.5%, yielding ~3.5 mg of purified enzyme from 900 g of fetal porcine skin. The enzyme is more active at 30 °C than at 37 °C and has a pH optimum near 8.0. Both catalase and bovine serum albumin are required by the enzyme for maximum activity. The sulfhydryl reagents *p*-(chloromercuri)-benzoate, *N*-ethylmaleimide, and iodoacetamide are potent inhibitors of the enzyme, whereas dithiothreitol appears to be an activator.

Collagen hydroxylysine and hydroxyproline residues are the result of posttranslational modification of the collagen polypeptides. Previous studies have demonstrated that these modifications of the collagen polypeptide are carried out by two separate enzymes, lysyl hydroxylase and prolyl hydroxylase (Miller, 1971; Kivirikko & Prockop, 1972; Popenoe & Aronson, 1972). Both of these enzymes require molecular oxygen, α-ketoglutarate, ferrous iron, and ascorbic acid as cofactors (Cardinale & Udenfriend, 1974). Further progress has been made in the purification of lysyl hydroxylase from chick embryos (Ryhänen, 1976; Turpeenniemi et al., 1977); however, it appears that the enzyme used in those studies was less than 40% pure. The hydroxylation of collagen lysine residues is a required prerequisite to the glycosylation of collagen (Butler & Cunningham, 1966) and to the formation of certain cross-link components that contain hydroxylysine residues (Tanzer, 1973; Barnes et al., 1971; Mechanic, 1972).

We wish to report the purification of fetal porcine skin lysyl hydroxylase to greater than 95% purity and some of the enzymatic properties of the enzyme. Relatively simple techniques

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Table I: Purification of Lysyl Hydroxylase[a]

fraction[b]	vol (mL)	mg of protein per mL	total act.[c] (units)	sp act. (cpm of THO per mg of protein)	purifn factor	recovery of act. (%)
SF-16	3400	5.61	19.0×10^3	253	1.0	100
AS-70	301	10.06	10.8×10^3	582	2.3	56.6
DC	78	8.2	4.7×10^3	1859	7.4	24.7
NE-I	10.2	0.79	0.27×10^3	8597	34.0	1.4
NE-II	6.9	0.50	0.48×10^3	35091	139	2.5

[a] 900 g of fetal porcine skin. [b] The fraction designations are the ones used under Materials and Methods: SF-16, 16000*g* supernatant fraction; AS-70, 0–70% ammonium sulfate precipitation fraction; DC, the DEAE-cellulose pooled fractions; NE-I and NE-II, the aminoethyl-Sepharose 4B pooled fractions I and II, respectively. [c] One unit of enzyme activity is equal to the counts per minute of THO formed per milligram of protein per hour.

involving ammonium sulfate fractionation, DEAE-cellulose chromatography, and aminoethyl-Sepharose 4B chromatography were used in the purification of the enzyme.

Materials and Methods

Ammonium Sulfate Fractionation. Nine hundred grams of fetal pig skin (Pel-Freez) was minced with scissors and homogenized in 100-g batches in 250 mL of homogenization buffer (0.05 M Tris-HCl, 10^{-5} M EDTA,[1] 2×10^{-4} M GSH, and 50 μg/mL PMSF, pH 7.5, at 5 °C) by use of a Polytron homogenizer at top speed for 1.5 min. The homogenate was centrifuged at 16000*g* for 15 min and the supernatant fraction, SF-16, decanted (3400 mL). The SF-16 was adjusted to 70% saturation by addition of ammonium sulfate over a 30-min period with stirring. Stirring was continued for an additional 15 min before centrifugation at 16000*g* for 15 min. The resultant pellets were resuspended in 200 mL of dialysis buffer, homogenization buffer minus PMSF, and then dialyzed against 4 L of dialysis buffer by use of an Isco rocking dialyzer. The final volume of the AS-70 fraction after dialysis and centrifugation at 16000*g* for 15 min was 301 mL.

DEAE-cellulose Chromatography. A 2.5 × 80 cm column of DE-52 (Whatman) was prepared and equilibrated with dialysis buffer. A 300-mL aliquot of the AS-70 was pumped onto the column at 100 mL/h. The column was washed with 300 mL of dialysis buffer, and bound proteins were eluted with a 2-L linear gradient of NaCl (0–0.5 M) in dialysis buffer at a rate of 100 mL/h. Thirty-milliliter fractions were collected. The effluent was monitored for absorbance at 280 nm by an Isco UV Type VI monitor using a 3-mm flow cell. Those fractions with the highest specific activity were combined, and the enzyme was concentrated by precipitation and dialyzed as described above for the AS-70 preparation. The final volume of this fraction, referred to subsequently as DC, was 78 mL.

Preparation of Aminoethyl-Sepharose 4B. To 100 mL of moist Sepharose 4B which had been washed with 500 mL of deionized water was added 100 mL of deionized water and 25.0 g of cyanogen bromide. The pH of the solution was maintained at 11 by the addition of 5 N NaOH, and the temperature was maintained at ~25 °C by the addition of ice. Once the pH began to stabilize, ~15 min, the mixture was filtered by use of a course scintered glass funnel and the Sepharose was washed with 1 L of 0.1 M $NaHCO_3$ (pH 10.0). One hundred milliliters of the bicarbonate buffer containing 13.5 mL of ethylenediamine was added to the activated Sepharose 4B and the mixture stirred overnight at 5 °C. The aminoethyl-Sepharose 4B was washed with 500 mL of 1.0 M NaCl, with 500 mL of deionized water, and finally with 500 mL of dialysis buffer. The aminoethyl-Sepharose 4B was poured into a 2.5-cm diameter column equipped with flow adaptors and equilibrated with dialysis buffer.

Aminoethyl-Sepharose 4B Chromatography. Seventy-seven milliliters of DC lysyl hydroxylase was pumped at 1.0 mL/min onto a 2.5 × 28 cm column of aminoethyl-Sepharose 4B that had been equilibrated with dialysis buffer. The column was washed with dialysis buffer at 1.0 mL/min until the unbound proteins were eluted as evidenced by the absorbance of the effluence at 280 nm approaching base line. Bound proteins were eluted from the column by a 1-L linear gradient of NaCl (0–0.2 M) in dialysis buffer with a flow rate of 1.0 mL/min. Ten-milliliter fractions were collected and assayed for lysyl hydroxylase activity.

Polyacrylamide Gel Electrophoresis. Native Gels. The polyacrylamide gels consisted of a 4% acrylamide stacking gel and a 6% acrylamide running gel (Davis, 1964). Duplicate gels were run with the purified lysyl hydroxylase. One gel was stained in a solution of 0.02% Coomassie Brilliant Blue R250, 7% glacial acetic acid, 5% trichloroacetic acid, and 20% methanol and destained in an aqueous solution of 7% glacial acetic acid and 20% methanol. The other gel was sliced into 2-mm slices, and each slice was added to the 1.0-mL assay and incubated at 30 °C for 5 h before vacuum distillation of the tritiated water to determine enzymatic activity.

Assay for Lysyl Hydroxylase Activity. Enzymatic activity was determined by the tritium-release assay (Miller, 1971) using L-[4,5-$^3H_2$]lysine-labeled protocollagen prepared from chick calvaria (Miller, 1975). The assay volume was 1.0 mL and consisted of the following: Tris-HCl (pH 7.8 at 30 °C), 50 μmol; α-ketoglutarate, 0.5 μmol; sodium ascorbate, 0.5 μmol; ferrous ammonium sulfate, 0.1 μmol; bovine serum albumin, 0.5 mg; catalase, 0.2 mg; [4,5-$^3H_2$]lysyl-labeled protocollagen, 10 μg (~100 000 cpm); lysyl hydroxylase, as indicated in the text. Incubations were for 1 h at 30 °C or as indicated in the text. The amount of enzymatic activity was determined by vacuum distillation and counting the tritiated water formed as previously described (Miller, 1971).

Results

As indicated in Table I, 19.0×10^3 units of lysyl hydroxylase activity was present in the initial extract (SF-16) of 900 g of fetal porcine skin. The SF-16 had a specific activity of 253 cpm of THO per mg of protein.

Ammonium sulfate fractionation of the proteins in the SF-16 in the range of 0–70% saturation resulted in ~57% recovery of activity with a 2.3-fold purification. Attempts to make additional fractionations between 0 and 70% saturation resulted either in precipitation of little protein or in significant precipitation of lysyl hydroxylase activity with about the same specific activity found in the 0–70% cut.

[1] Abbreviations used: GSH, glutathione; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

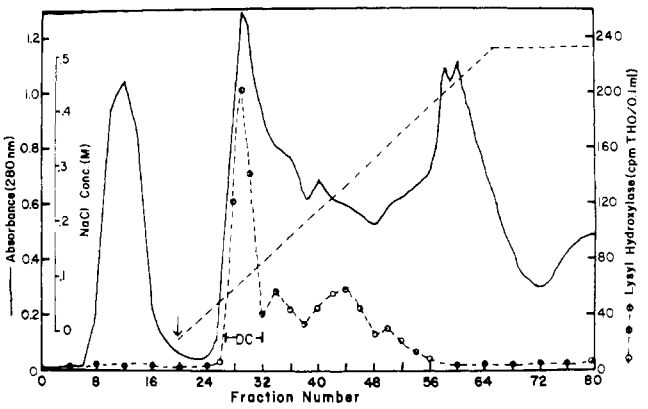

FIGURE 1: DEAE-cellulose chromatography. The chromatography was carried out as indicated under Materials and Methods. Salt gradient elution was begun at the point indicated by the arrow. (—) Absorbance at 280 nm; (O) lysyl hydroxylase activity; (---) NaCl concentration (molar) in buffer being pumped onto the column. |—DC—| indicates the fractions that were pooled and saved for further purification.

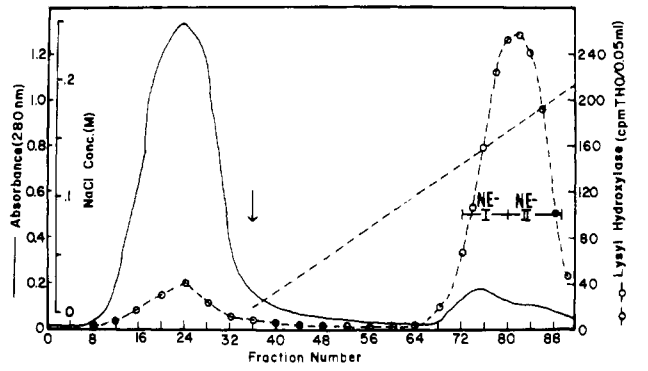

FIGURE 2: Aminoethyl-Sepharose 4B chromatography. The chromatography was carried out as indicated under Materials and Methods. The arrow indicates the point at which salt gradient elution was begun. (—) Absorbance at 280 nm; (O) lysyl hydroxylase activity; (---) NaCl concentration (molar) in buffer being pumped onto the column. NE-I and NE-II indicate fractions that were pooled and further analyzed for lysyl hydroxylase activity.

In our earlier report on the separation of lysyl hydroxylase from prolyl hydroxylase, DEAE-Sephadex chromatography was used. Soon afterwards DEAE-cellulose was found to be just as effective in the separation of these enzymes, and it did not present the shrinkage problem associated with the Sephadex available at that time. The DC pooled fraction obtained from DEAE-cellulose chromatography (Figure 1) of the AS-70 fraction contained ~25% of the lysyl hydroxylase activity originally present in the SF-16 (Table I). Also as indicated in Table I, the DC fraction was approximately 7.4-fold purified when compared to the SF-16.

Attempts to purify lysyl hydroxylase by affinity chromatography on columns of protocollagen coupled to Sepharose 4B by use of the spacer ethylenediamine have yielded variable results. However, in the course of these studies, it was observed that lysyl hydroxylase binds in a highly specific manner to ethylenediamine coupled to Sepharose 4B (NE-Sepharose 4B).

As indicated in Figure 2, most of the enzymatic activity that was eventually recovered from the NE-Sepharose 4B column bound to the column and was eluted with a NaCl gradient. Two pooled fractions of purified lysyl hydroxylase were obtained from the column, NE-I and NE-II. The NE-I fraction contained 1.4% of the original activity with a 34-fold purification, and the NE-II fraction contained 2.5% of the original activity with a 139-fold purification (Table I). Only ~20% of the lysyl hydroxylase activity applied to the NE-Sepharose 4B column was recovered. Further activity could not be obtained by elution of the column with higher concentrations of NaCl. Low recovery of activity is probably due to the tendency of the enzyme to aggregate with concomitant loss of activity.

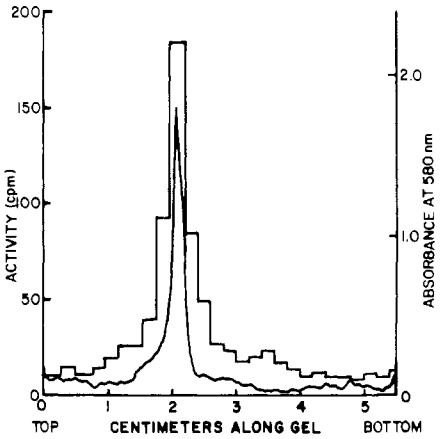

FIGURE 3: Polyacrylamide gel electrophoresis of lysyl hydroxylase under nondenaturing conditions. Electrophoresis and assay for lysyl hydroxylase activity were carried out as indicated under Materials and Methods. The line graph represents the 580-nm scan of a gel run with 20 μg of the NE-II fraction of lysyl hydroxylase. The bar graph represents the activity profile of a companion gel run with 50 μg of lysyl hydroxylase.

The most purified enzyme fraction, NE-II, yielded a single Coomassie Blue staining band upon polyacrylamide gel electrophoresis and therefore appeared to be homogeneous (Figure 3). Furthermore, when a duplicate gel was sliced and the slices were assayed for lysyl hydroxylase activity, activity coincided with the Coomassie Blue staining band (Figure 3). In some preparations there was a minor protein contaminant in the NE-II fraction that migrated more slowly on polyacrylamide gels than did the lysyl hydroxylase. Polyacrylamide gel electrophoresis of the NE-I fraction usually yielded two or three bands, in addition to the lysyl hydroxylase band (data not shown). Approximately 3.5 mg of purified lysyl hydroxylase (NE-II) was obtained from 900 g of fetal porcine skin.

Electrophoresis of the purified enzyme (NE-II fraction on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels, after denaturation by use of sodium dodecyl sulfate and β-mercaptoethanol (Laemmli, 1970), yielded two Coomassie Blue staining bands. The molecular weights of these polypeptides were estimated to be 70000 and 115000 by the use of β-galactosidase, phosphorylase α, transferrin, bovine serum albumin, and ovalbumin as standards on the $NaDodSO_4$ gels.

Assays of the lysyl hydroxylase at 30 and 37 °C indicate that greater activity is obtained at 30 °C (Figure 4). The activity is directly proportional to the mount of enzyme added after an apparent lag in activity at the lower levels of enzyme assayed. At the lower levels of enzyme assayed, there is little difference in activity at the two temperatures; however, when higher levels of enzyme were assayed approximately twice as much activity was obtained at 30 °C as at 37 °C. Partially purified preparations of chick embryo lysyl hydroxylase appear to be more active at 37 °C than at 30 °C (Popenoe & Aronson, 1972).

The same substrate preparation was used throughout these studies. With this substrate preparation, the amount of tritiated water formed was directly proportional to the amount of substrate added to the assay, up to ~800 cpm (Figure 5). Beyond this point the ratio of tritiated water formed to the amount of substrate added decreased, suggesting that enzyme became a limiting factor.

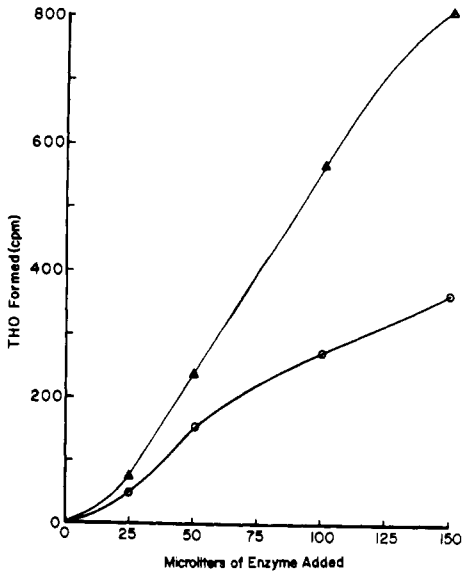

FIGURE 4: Assay of lysyl hydroxylase at 30 and 37 °C for activity. The enzyme preparation contained 20 μg of purified lysyl hydroxylase per mL. Incubations were for 1 h. (Δ) 30 °C; (O) 37 °C.

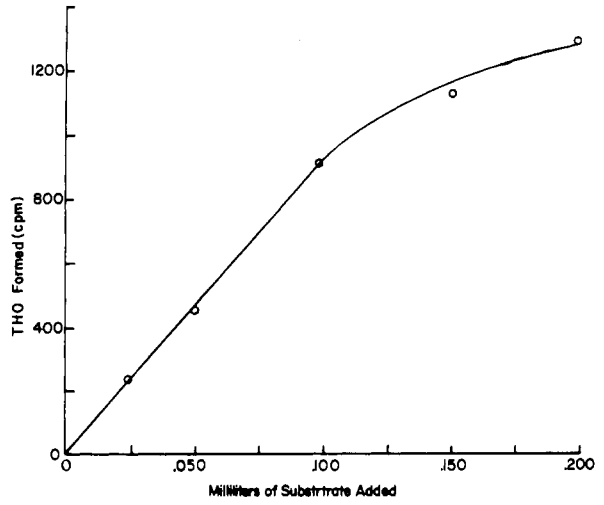

FIGURE 5: Lysyl hydroxylase activity vs. the amount of protocollagen substrate added to the assay. The substrate preparation contained 107 μg of protocollagen per mL as determined by the collagenase assay of Peterkofsky & Diegelmann (1971) as modified by Murray et al. (1977). The assays contained 2 μg of purified lysyl hydroxylase.

There is disagreement as to what is the pH optimum for partially purified lysyl hydroxylase from chick embryos. One study indicates a pH optimum of 7.4 (Kivirikko & Prockop, 1972), and another indicates a pH optimum of 8.0 (Popenoe & Aronson, 1972). With the purified porcine lysyl hydroxylase, a pH optimum of 8.0 at 30 °C was obtained (Figure 6).

Previous studies with lysyl hydroxylase have indicated that both bovine serum albumin and catalase are essential for maximum activity. In one study (Kivirikko & Prockop, 1972), with lysyl hydroxylase from chick embryos, omission of bovine serum albumin from the assay resulted in a 38% decrease in activity, whereas, the omission of both bovine serum albumin and catalase resulted in a 40% decrease in activity. In another study (Miller, 1972), with lysyl hydroxylase from fetal rat skin, the omission of either bovine serum albumin or catalase resulted in a 2 to 3% decrease in activity, wheres the omission of both bovine serum albumin and catalase resulted in a 38% decrease in activity. The earlier studies have used partially purified lysyl hydroxylase. In the present studies, with highly

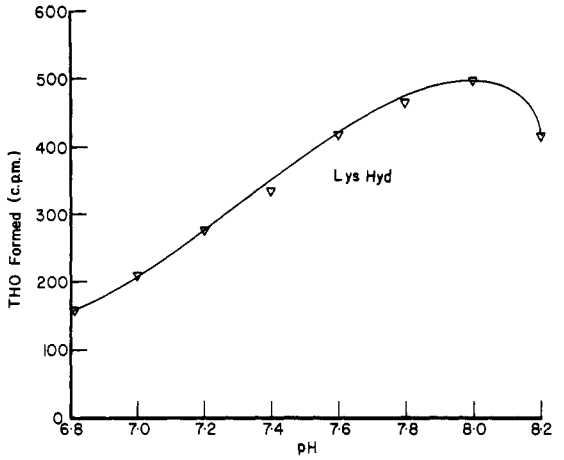

FIGURE 6: Effect of pH on lysyl hydroxylase activity. The assays contained 50 μmol of Tris-HCl adjusted to the indicated pH before addition of 2 μg of purified lysyl hydroxylase. Incubations were for 1 h.

Table II: Effect of Bovine Serum Albumin and Catalase on Lysyl Hydroxylase Activity

mg of BSA per assay	mg of catalase per assay	THO formed (cpm)	% of control
0	0.2	416	69.8
0.5	0.2	596	100
1.0	0.2	482	80.9
2.0	0.2	379	63.6
4.0	0.2	270	45.3
0	0	205	34.9
0.5	0	303	50.8
0.5	0.05	405	68.0
0.5	0.10	564	94.6
0.5	0.2	589	98.8
0.5	0.4	577	96.8

Table III: Effect of Sulfhydryl Reagents on Purified Porcine Skin Lysyl Hydroxylase

sulfhydryl reagent[a]	concn (M)	% inhibn
p-(chloromercuri)benzoate	10^{-3}	97.4
	10^{-4}	97.8
	10^{-5}	17.7
N-ethylmaleimide	10^{-3}	81.4
	10^{-4}	48.7
	10^{-5}	5.8
iodoacetamide	10^{-3}	80.6
	10^{-4}	37.3
	10^{-5}	6.0

[a] The sulfhydryl reagents were added to the complete assay minus substrate. After a 5-min incubation at 30 °C, substrate was added and incubation continued for another hour.

purified porcine skin lysyl hydroxylase, a 30% decrease in activity by omission of bovine serum albumin, a 50% decrease in activity by omission of catalase, and a 65% decrease in activity by omission of both bovine serum albumin and catalase from the assay were obtained (Table II). Thus, it appears that the purified porcine lysyl hydroxylase is more dependent upon catalase and bovine serum albumin for maximum activity than was the impure preparation of fetal rat skin lysyl hydroxylase used in previous studies (Miller, 1972).

The sulfhydryl reagents *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, and iodoacetamide inhibit lysyl hydroxylase (Table III). Of the three compounds, *p*-(chloromercuri)-benzoate is the most potent inhibitor. Lysyl hydroxylase is almost completely inhibited by *p*-(chloromercuri)benzoate at 10^{-4} M, whereas *N*-ethylmaleimide and iodoacetamide inhibit

Table IV: Effect of Dithiothreitol on Purified Porcine Skin Lysyl Hydroxylase

[dithiothreitol] (M)[a]	lysyl hydroxylase act. (% of control)
0	100[b]
10^{-2}	142
10^{-3}	96
10^{-4}	97
10^{-5}	103
10^{-6}	100

[a] Lysyl hydroxylase was preincubated with DTT (total volume 40 μL) for 5 min at 30 °C before the other reagents were added. [b] Lysyl hydroxylase was preincubated as above, except no DTT was added.

by ~50% at 10^{-4} M. These data on *p*-(chloromercuri)-benzoate inhibition of purified lysyl hydroxylase are consistent with previous studies performed with partially purified enzyme (Kivirikko & Prockop, 1972). Thus, it appears that free sulfhydryl groups are essential for lysyl hydroxylase to be enzymatically active.

Prolyl hydroxylase, the enzyme that hydroxylates certain collagen proline residues, is inactivated by DTT (Popenoe et al., 1969; Berg & Prockop, 1973; Stassen et al., 1973). This suggests that disulfide bonds are necessary for enzymatic integrity of the enzyme. It has been shown (Berg & Prockop, 1973) that 10 mM DTT dissociates the tetrameric enzyme into inactive dimers and monomeric subunits. In contrast to the data presented on prolyl hydroxylase, it appears that lysyl hydroxylase is activated by DTT at a concentration of 10 mM (Table IV). Lower concentrations of DTT had little or no effect on lysyl hydroxylase. These studies indicate that disulfide bonds are not essential for lysyl hydroxylase to be enzymatically active, even though they play a critical role in prolyl hydroxylase.

Discussion

In many tissues lysyl hydroxylase appears to be associated with cellular membranes. However, increased amounts of lysyl hydroxylase activity were not obtained from the fetal porcine skin by the inclusion of 0.1% Triton X-100 in the homogenization buffer. Lysyl hydroxylase is a relatively unstable enzyme and readily forms high molecular weight aggregates (Kivirikko & Prockop, 1972; Rhyänen, 1976). The heterogeneous nature of the activity profile obtained in the DEAE-cellulose (Figure 1) of the enzyme is probably due to aggregation. This may also account for the low recovery of activity (~20%) upon NE-Sepharose 4B chromatography of the enzyme. The observation that the porcine enzyme binds to NE-Sepharose 4B in a highly specific manner has provided us with an essential method for the purification of the enzyme to homogeneity. Studies with polyacrylamide gel electrophoresis in which one of two companion gels was stained for protein and the other sliced and assayed for lysyl hydroxylase activity indicated that activity comigrated with the one protein staining band.

The purified porcine lysyl hydroxylase is more active at 30 than 37 °C and has a pH optimum near 8.0. It also has a greater requirement for bovine serum albumin and catalase than has been reported for other preparations of lysyl hydroxylase. These differences are likely due to differences in species and tissues from which the enzyme was obtained or to the lower state of purity of the earlier enzyme preparations.

The sulfhydryl reagents *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, and iodoacetamide are highly inhibitory to porcine lysyl hydroxylase. *p*-(Chloromercuri)benzoate at 10^{-4} M almost completely inhibits enzymatic activity. In contrast to prolyl hydroxylase, which is converted to inactive momers and dimers by 10 mM DTT, lysyl hydroylase is activated or stabilized by 10 mM DTT.

The porcine skin lysyl hydroxylase consists of two subunits of 70 000 and 115 000 daltons. Thus, the enzyme appears to be quite different from the lysyl hydroxylase partially purified from chick embryos (Turpeenniemi et al., 1977). The chick embryo lysyl hydroxylase appears to consist of one subunit with a molecular weight of 87 000. We have recently obtained highly purified lysyl hydroxylase from fetal bovine skin (H. H. Varner, Jr., and R. L. Miller, unpublished experiments). This enzyme appears to contain a single subunit with a molecular weight of 88 000. Thus, it is apparent that there are two different molecular forms of lysyl hydroxylase. This is interesting in that prolyl hydroxylases are very similar in molecular weight and subunit composition regardless of the species or tissue from which they have been isolated.

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