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METALLOPORPHYRIN CHELATASE FROM BARLEY

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SUMMARY

1. A soluble enzyme which catalyzes the insertion of both zinc and iron into porphyrins has been prepared from etiolated barley seedlings. Purification of about 30-fold has been achieved by $(NH_4)_2SO_4$ fractionation and chromatography on Sephadex G-150. Both zinc-chelatase and ferrochelatase activity fractionated in a parallel manner during all steps of purification.

2. A simple and rapid procedure for determination of zinc-chelatase activity is described. This is based on fluorimetric determination of the rate of formation of zinc protoporphyrin.

3. Evidence both favorable and opposed to the view that a single enzyme is involved in both iron- and zinc-chelatase action is discussed. Ferrochelatase activity was stimulated by preincubation with glutathione and dithoithreitol and was inhibited by *p*-chloromercuribenzoate and iodoacetamide. Zinc-chelatase activity was stimulated by ATP and was relatively insensitive to air. Both activities had the same pH-activity curve, and Fe^{2+} competitively inhibited zinc-chelatase activity.

INTRODUCTION

The extensive involvement of metalloporphyrins in a variety of biological oxidations gives paramount interest to the means of their biosynthesis. It is commonly accepted that a step in this pathway is the enzymatic insertion of a metal into the free porphyrin. Enzymes which catalyze such a step have been reported from pig and rat liver[1–3], avian erythrocytes[4,5], yeast[6], and several microorganisms[7–9]. Comparable enzymes from higher plants have not been extensively studied, although LITTLE and coworkers[10,11], have briefly reported on a metal chelatase from barley, and JONES[12,13] has described a ferrochelatase from spinach. PORRA AND LASCELLES[14] have recently investigated the occurrence of ferrochelatase in chloroplasts and mitochondria of plants. The present paper describes the partial purification of a metalloporphyrin chelatase from etiolated barley seedlings and compares some of its properties with those of enzymes previously studied.

Enzymes which catalyze the chelation of iron by protoporphyrin are classified as protohaem ferrolyase (EC 4.99.1.1). Some authors have used trivial names such as ferrochelatase[2] and iron-protoporphyrin chelatase[1] for enzymes showing such activity. The enzyme from barley catalyzes the formation of both zinc and iron porphyrins. Since it has not been established unequivocally that a single enzyme catalyzes both reactions, the term 'zinc-chelatase activity' has been used in this paper to designate activity observed when zinc and protoporphyrin served as substrates. The term 'ferrochelatase activity' has been employed when the insertion of iron into porphyrin was studied.

METHODS AND MATERIALS

Purification of the enzyme

The purification procedure described below was used to prepare protein fractions for study of either zinc- or ferrochelatase activity. This procedure was repeated many times and gave essentially the same relative results each time for both types of activities.

All operations were performed at 5°, and Tris–HCl buffer, 0.1 M (pH 8.0), was employed throughout, unless otherwise noted.

The enzyme was isolated from 6-day-old etiolated barley seedlings which had been grown in the dark at 25–28° in glazed pots containing vermiculite and water.

Extraction. The barley leaves were homogenized for 2 min in a Waring blender in a volume of buffer twice their weight. The homogenate was filtered through cheesecloth, and the filtrate (Fraction I) was centrifuged at 20 000 × *g* for 20 min. The resultant 'soluble' fraction (Fraction II) contained approx. 50% of the enzyme activity present in the filtered extract. Further centrifugation of Fraction II for 1 h at 105 000 × *g* did not remove more than 10% of the zinc-chelatase activity. The 'insoluble' fraction (Fraction III) obtained from centrifugation of Fraction I was estimated to contain at least 25% of the enzyme activity, but attempts to efficiently solubilize the zinc-chelatase activity by use of various detergents were not successful.

$(NH_4)_2SO_4$ fractionation. The $(NH_4)_2SO_4$ concentration of Fraction II was brought to 45% saturation by gradual addition of the crystalline salt. The solution was stirred for 15 min and the precipitate was removed by centrifugation and discarded. Additional solid $(NH_4)_2SO_4$ was added until 60% saturation was reached. The precipitate was collected by centrifugation and was dissolved in sufficient buffer to give a protein concentration of 15–20 mg per ml. This fraction, which contained most of the observable enzymatic activity, was dialyzed overnight against Tris buffer. The dialyzed sample, Fraction IV, could be stored at −15° for several months without appreciable loss of activity.

Sephadex column chromatography. Fraction IV was purified further by gel filtration. For this, the enzyme (50–75 mg protein) was dissolved in 5 ml of buffer and was applied to a column (2.5 cm × 40 cm) which had been packed with Sephadex G-150 previously equilibrated with Tris–HCl buffer, 0.1 M (pH 8.0). Elution was accomplished with the same buffer, the eluate being collected in 2.5-ml fractions at a flow rate of 0.2 ml per min. Fractions with the highest enzymatic activity were pooled and then freeze-dried. The resultant dry white powder maintained enzymatic activity for several months when stored at −15°. For assay, the powder was redis-

solved in 0.01 M Tris buffer to give a clear, pale yellow solution (Fraction V) containing 1–3 mg protein per ml.

Enzyme assay

Three standard assay procedures to determine metal-chelatase activity have been employed. Unless noted otherwise, all the studies reported have been done with partially purified enzyme (Fraction V).

Zinc-chelatase activity

Spectrophotometric assay. In this assay the amount of zinc protoporphyrin formed was estimated spectrophotometrically. The reaction mixture (1.0–1.1 ml) contained 90 nmoles of protoporphyrin, 100 nmoles of $ZnCl_2$, 2.5 mg of ATP, 80 μmoles of Tris–HCl buffer, (pH 8.0), *plus* 0.4 ml of enzyme. The enzymatic reaction was initiated by addition of protoporphyrin. Incubation was carried out in unshaken test tubes (12 mm × 100 mm) at 40° for 1 h. After the incubation period, the porphyrins were extracted from the reaction mixture with ether and the nmoles of zinc protoporphyrin formed per assay mixture were calculated from the absorbance at 404 and 415 nm, as described by NEUBERGER AND TAIT[7]. In this assay, the unit of activity was defined as the amount of enzyme catalyzing the formation of 1 nmole of zinc protoporphyrin per min. Specific activity was expressed as the number of units of activity per mg of protein.

Fluorimetric assay. In this assay the increase in fluorescence at 585 nm due to the formation of metalloporphyrin was used to estimate zinc-protoporphyrin production. The reaction mixture (1.0–1.1 ml) contained 90 nmoles of protoporphyrin, 100 nmoles of $ZnCl_2$, 2.5 mg ATP, 80 μmoles of Tris–HCl buffer, (pH 8.0), *plus* 0.4 ml enzyme. This was incubated in unshaken test tubes (12 mm × 100 mm) at 40° for 4 min. The reaction was initiated by addition of protoporphyrin, and then 0.1-ml aliquots were removed at zero time and after 4 min of incubation. The two aliquots were added to 3.0-ml portions of acetone–0.1 M NH_4OH (9:1, v/v) and the resultant solution centrifuged to remove the turbidity. The difference in fluorescence at 585 nm (excitation radiation 400 nm) of the clear acetone solutions was observed and the amount of zinc protoporphyrin was estimated by comparison of this difference with the fluorescence of comparable solutions containing known concentrations of zinc protoporphyrin. A unit of enzyme activity was defined as that amount of enzyme catalyzing the formation of 1 nmole of zinc protoporphyrin per min. Specific activity was expressed as the number of units of activity per mg of protein.

Ferrochelatase activity

In this assay spectral observation of the pyridine hemochromogen was used to estimate iron-porphyrin formation. Mesoporphyrin was used as substrate in the standard assay since this porphyrin was a better substrate for ferrochelatase activity than was protoporphyrin. Just prior to assay, the enzyme, dissolved in Tris buffer, was preincubated with 5.3 mM glutathione for 20 min. The preincubated enzyme was then assayed in the standard reaction mixture which contained the following total amounts in a final volume of 4.0 ml: 200 nmoles of mesoporphyrin, 400 nmoles $FeSO_4$, 20 μmoles of glutathione, 320 μmoles of Tris–HCl buffer, (pH 8.0), and enzyme (1–3 mg protein). The reaction mixture was incubated *in vacuo* in Thunberg tubes which had been flushed three times with N_2. After a 1-h incubation at 40°, the reaction was terminated by placing the tubes in an ice bath, after which the amount of iron

porphyrin was estimated by observing the difference spectrum of the oxidized and reduced pyridine hemochromogen[2]. As suggested by PORRA *et al.*[15], iodoacetate (0.25 mmoles) was added to the Thunberg tubes as soon as their contents were exposed to air. Activity of the enzyme was expressed as the nmoles of iron mesoporphyrin formed per incubation mixture in 1 h, and a unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmole of mesohaem per min. Specific activity was expressed as the number of units of activity per mg protein.

Materials and methods

Protoporphyrin IX was prepared by either acid or alkaline hydrolysis[16] of protoporphyrin dimethyl ester prepared by the method of GRINSTEIN[17]. Deuteroporphyrin IX was prepared by acid hydrolysis of its dimethyl ester which was prepared according to CHU AND CHU[18]. Coproporphyrin III was isolated from the culture medium of *Rhodopseudomonas spheroides* grown on medium IS supplemented with 1.0 mM ethionine[19]. Pierce Chemical supplied mesoporphyrin IX and its dimethyl ester, while Nutritional Biochemical supplied hematoporphyrin IX. Free porphyrins were dissolved in 0.1 M NaOH and then diluted with water and ethanol to give a 0.02 M NaOH–ethanol solution (1:1, v/v). Solutions of the porphyrin dimethyl esters were prepared as described by PORRA AND JONES[8].

Absorbance measurements were made either with a Beckman DU-2 spectrophotometer or with a Cary Model-14 recording spectrophotometer. Fluorescence was measured with an Aminco Bowman spectrofluorometer. Protein was determined by the method of LOWRY *et al.*[20] with crystalline bovine albumin used as the standard.

RESULTS

Purification of the enzyme

Table I summarizes data for a single preparation of metal chelatase from etiolated barley leaves as described in METHODS AND MATERIALS. The data show that about 50% of the enzyme activity in the homogenate was observed in the soluble fraction. Although only partial recovery of activity was obtained in the $(NH_4)_2SO_4$

TABLE I

PURIFICATION OF METAL CHELATASE

Data are for a single enzyme preparation. Zn refers to zinc-chelatase activity which was measured fluorimetrically. Fe refers to ferrochelatase activity which was measured by the standard procedure.

Fraction	*Total units*		*Specific activity* × 10^2		*Yield (%)*		*Purification*	
	Zn	*Fe*	*Zn*	*Fe*	*Zn*	*Fe*	*Zn*	*Fe*
I. Homogenate	560	117	8.0	1.85	—		1.00	1.00
II. Soluble	280	53.5	7.0	1.33	51	46	0.80	0.72
III. Insoluble	-	30.0	—	2.12	—	26	—	1.15
IV. 45–60% $(NH_4)_2SO_4$	74	14.6	31	5.20	13	12	3.50	2.80
V. Sephadex fraction	35	7.5	200	44.3	6	6	23.0	23.7

fraction, negligible activity could be found in the discarded fractions. Possibly endogenous proteins concentrated in these latter fractions were inhibitory to residual enzymatic activity. In this experiment both the zinc- and iron-chelatase activities were purified to very similar degrees in the various fractions to yield a final purification of 23-fold. Other preparations have yielded up to 33-fold purification of zinc-chelatase activity. Further evidence that the two activities are fractionated in a parallel manner is given in Fig. 1, which shows the elution pattern for zinc- and iron-

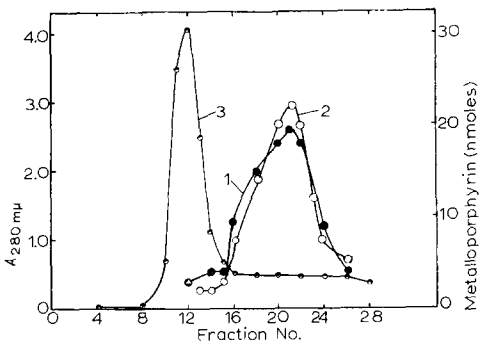


Fig. 1. Elution pattern for metal chelatase (Fraction IV) on Sephadex G-150. Curve 1, zinc-chelatase activity measured spectrophotometrically; Curve 2, ferrochelatase activity measured by the standard assay; Curve 3, absorbance at 280 nm.

chelatase activity when the enzyme was passed through Sephadex G-150. In this experiment the effluent volume for much of the extraneous protein corresponded approximately to the void volume of the column, while both enzymatic activities were eluted at very comparable rates in fractions corresponding to 1.5 to 2.0 times the void volume. The rate at which proteins are eluted from Sephadex columns has been used to estimate their molecular weight[21]. A single experiment with Sephadex G-150 in which four standard proteins were used as markers indicated that zinc-chelatase activity exhibited a molecular weight of about 55 000–65 000.

Evaluation of assay procedures

The study of enzymatic chelation of metals by porphyrins is difficult since the nonenzymatic rate is frequently significant and is dependent upon experimental conditions. Table II presents some information concerning the rate of enzymatic and nonenzymatic formation of zinc protoporphyrin. It is evident that, in the absence of ATP, nonenzymatic incorporation amounted to 13 nmoles of zinc protoporphyrin, while in the presence of boiled enzyme this value was 9.5 nmoles. The corresponding values in the presence of ATP were both zero. Since the standard assay included ATP, the latter value appears to be a more valid control for the assay of zinc chelatase. Boiled enzyme appeared to inhibit nonenzymatic incorporation of zinc into protoporphyrin. This is in accord with the observation that crystalline egg albumin, serum albumin, and ribonuclease at concentrations of 1–5 mg/ml were found to be strongly inhibitory to the nonenzymatic incorporation of zinc.

That the increase in fluorescence is a valid and quantitative measure of enzymatic formation of zinc protoporphyrin is evidenced by the following observations.

TABLE II

ENZYMATIC AND NONENZYMATIC FORMATION OF ZINC PROTOPORPHYRIN

The figures are those typically obtained for the amount of zinc protoporphyrin formed after incubation for 1 h under the conditions indicated. The reaction mixture was the same as that used for the spectrophotometric assay for zinc-chelatase except that the enzyme (0.8 mg protein, Fraction IV) and ATP (2.5 mg) were added only as indicated. The boiled enzyme was heated at 100° for 1 min.

Addition	*Zinc protoporphyrin (nmoles per assay)*	
	+ATP	*−ATP*
None	0	13
Boiled enzyme	0	9.5
Enzyme	55	30

When the enzymatic activity of a reaction mixture was measured both spectrophotometrically and fluorimetrically, the rate of increase in zinc protoporphyrin formation, as assayed spectrophotometrically, closely paralleled the rate of increase of fluorescence at 585 nm. In the fluorimetric assays reported, the concentration of zinc protoporphyrin varied from 0–1.5 μM. At these concentrations the fluorescence at 585 nm was found to be linearly proportional to the concentration of zinc protoporphyrin. Furthermore, appropriate control studies failed to reveal any quenching or enhancement of fluorescence caused by ATP, Zn^{2+}, Co^{2+} and Fe^{2+} when these agents were added to zinc protoporphyrin at the maximal concentrations employed in the enzymatic studies.

The fluorescence at 585 nm observed in the fluorimetric assay for zinc-chelatase activity was found to be dependent both on the concentration of the enzyme and on the period of incubation. That is, fluorescence was linearly proportional to the amount of enzyme present when the concentration of enzyme was less than 1.0 mg of protein per assay. Furthermore, the rate of increase of fluorescence in these assays was constant for the first 6 min of incubation and then declined gradually during the succeeding 20 min.

The relative insensitivity of the spectrophotometric assay for zinc- chelatase required longer incubation times to be employed for this assay and, on the basis of 2-h time-course studies, an incubation period of 1 h was selected as the standard time for incubation.

Studies with the spectrophotometric assay for ferrochelatase showed that the rate of formation of mesohaem was constant during the first hour of incubation but that this rate decreased greatly during the second hour. Because of this, an incubation time of 1 h was selected for the standard assay. Under standard assay conditions, only a small amount of mesohaem (2–4 nmoles per assay) was formed in the absence of enzyme.

Effect of temperature and pH

The effect of temperature on enzyme activity has been studied by observation of zinc-chelatase activity. Activity was completely destroyed by heating the enzyme in Tris–HCl buffer, 0.1 M (pH 8.0), at 100° for 1 min. An Arrhenius plot of data

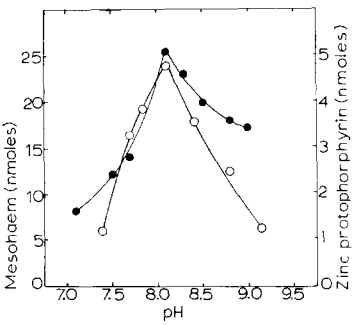


Fig. 2. Effect of pH on zinc-chelatase and ferrochelatase activity. Activities were measured in Tris–HCl buffer (0.1 M) which was adjusted to the indicated pH. ●—●, zinc-chelatase activity measured fluorimetrically and expressed as nmoles of zinc protoporphyrin formed in 4 min; ○—○, ferrochelatase activity measured by the standard assay and expressed as nmoles of mesohaem formed in 1 h.

showing the effect of incubation temperature (9–45°) on enzymatic incorporation of zinc into protoporphyrin gave an activation energy for this reaction of 10.9 kcal/mole. This value is comparable with the activation energy of 14.8 kcal/mole reported by NEUBERGER AND TAIT[7] for a metallochelatase from *R. spheroides*.

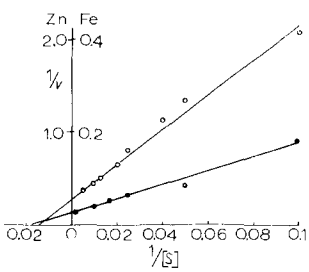
The effect of pH on both zinc- and iron-chelatase activity is given in Fig. 2. The pH optimum for both types of activity appears to be close to pH 8.1. Enzyme which had been adjusted to either pH 7.4 or 9.1 for 15 min and then assayed at pH 8.1 showed very low ferrochelatase activity. This would indicate that the barley enzyme was irreversibly inactivated at a pH not more than 1 unit removed from either side of the optimum pH for activity. The presence of the substrate, mesoporphyrin, did not appear to stabilize the enzyme against inactivation in this experiment.

Effect of substrate concentration

The effect of metal-ion concentration on chelatase activity is presented in Fig. 3 as a Lineweaver–Burk plot. These data show that, under the conditions employed, the apparent K_m for zinc insertion into protoporphyrin is 51 μM, while the apparent K_m for iron insertion into mesoporphyrin is 62 μM. Similar data concerning the effect of porphyrin concentration on metal-chelatase activity is given in Fig. 4. These data show that, under the conditions employed, the apparent K_m for protoporphyrin is 10 μM when zinc-chelatase activity is measured, and that the apparent K_m for mesoporphyrin is 25 μM when ferrochelatase activity is measured.

Specificity of enzyme

The enzyme catalyzed the formation of only zinc and iron porphyrins. All attempts to demonstrate fluorimetrically or spectrophotometrically the enzymatic formation of magnesium protoporphyrin in extracts from barley have been negative although many different extraction and incubation procedures have been tried. When nickel was substituted for zinc in the standard incubation mixture for assay of zinc-chelatase, no spectrophotometric evidence for the formation of a metalloporphyrin was obtained. Enzyme preparations with high zinc-chelatase activity also failed to



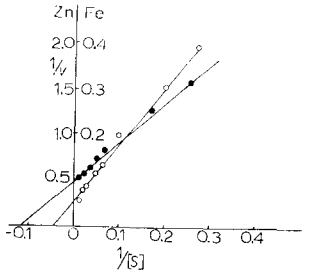


Fig. 3. Lineweaver-Burk plot of the effect of metal ion concentration on chelatase activity. ●—● (Zn), zinc-chelatase activity measured by the standard fluorimetric assay except that the concentration of zinc was varied. Substrate concentration ([S]) is expressed as μM zinc, and velocity (v) is expressed as nmoles of zinc protoporphyrin formed per min. ○ -○ (Fe), ferrochelatase activity measured by the standard assay except that the concentration of iron was varied. Substrate concentration ([S]) is expressed as μM iron and velocity (v) is expressed as nmoles of mesohaem formed per h.

Fig. 4. Lineweaver Burk plot of the effect of porphyrin concentration on chelatase activity. ●—● (Zn), zinc-chelatase activity measured by the standard fluorimetric assay except that the concentration of protoporphyrin was varied. Substrate concentration ([S]) is expressed as μM protoporphyrin, and velocity (v) is expressed as nmoles of zinc protoporphyrin formed per min. ○ -○ (Fe), ferrochelatase activity measured by the standard assay except that the concentration of mesoporphyrin was varied. Substrate concentration ([S]) is expressed as μM mesoporphyrin, and velocity (v) is expressed as nmoles of iron mesoporphyrin formed per h.

show measurable enzymatic insertion of cobalt into either protoporphyrin or mesoporphyrin. In these studies, the incubation conditions and the spectrophotometric assay of PORRA AND ROSS[9] were employed except that no detergent was used.

The specificity of the metal chelatases for different porphyrins has been studied by observation of the amount of iron porphyrin formed when various porphyrins were tested as substrates in the assay for ferrochelatase activity. The results tabulated in Table III show that, of the porphyrins tested, mesoporphyrin was the most effective substrate. Hematoporphyrin was fairly effective as a substrate while protoporphyrin and deuteroporphyrin were appreciably less effective. The dimethyl esters of these porphyrins were not utilized. Additional experiments have also failed to show enzymatic insertion of iron into coproporphyrin. Control experiments in which the

TABLE III

SPECIFICITY OF FERROCHELATASE ACTIVITY FOR PORPHYRIN

The enzyme was assayed for ferrochelatase activity under the standard conditions except that various porphyrins were substituted for mesoporphyrin.

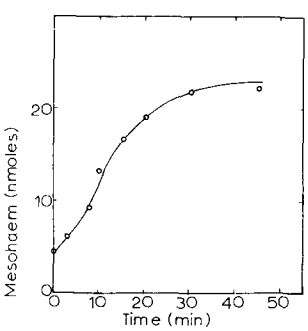
Porphyrin	*Activity (nmoles of iron porphyrin/h)*
Mesoporphyrin	29.4
Hematoporphyrin	23.9
Deuteroporphyrin	13.2
Protoporphyrin	11.8
Dimethyl esters of meso, deutero, or protoporphyrin	0

standard assay procedure for ferrochelatase activity were performed in the absence of enzyme showed that the amount of iron porphyrin formed nonenzymatically in the presence of the dicarboxylic porphyrins was less than 4.0 nmoles per assay.

Effect of O_2 and sulfhydryl compounds

Whereas O_2 inhibited and sulfhydryl compounds stimulated ferrochelatase activity, comparable effects on zinc-chelatase activity were not observed. The latter activity was found to be the same whether the incubation for the spectrophotometric assay was performed in unshaken tubes or in Thunberg tubes which had either been evacuated or flushed with N_2 several times. Furthermore, certain reducing compounds, when preincubated with the enzyme before its assay, failed to stimulate strongly zinc-chelatase activity. For example, 5.0 mM glutathione and dithiothreitol were inhibitory and ascorbic acid at the same concentration stimulated zinc-chelatase activity only 1.1–1.2-fold. In these studies the preincubation was performed aerobically, and the assay for chelatase activity was done with the standard spectrophotometric procedure except that the incubation of the enzymatic reaction was anaerobic.

As indicated in Fig. 5, preincubation of the enzyme with glutathione prior to its assay greatly stimulated ferrochelatase activity. Maximal activation, which was generally 4- to 5-fold that of the untreated sample, was attained after 20 min of preincubation. The degree of activation was found to be proportional to the glutathione concentration. Experiments in which the enzyme, dissolved in Tris–HCl buffer, 0.1 M (pH 8.0), was preincubated for 20 min with various concentrations of glutathione and then assayed for ferrochelatase activity showed that maximal activation was achieved by 5 mM glutathione. Similar activation was achieved with dithiothreitol which showed optimal activation at one tenth the concentration required for glutathione.



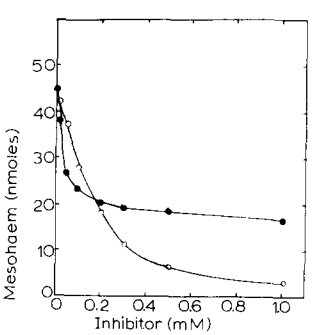


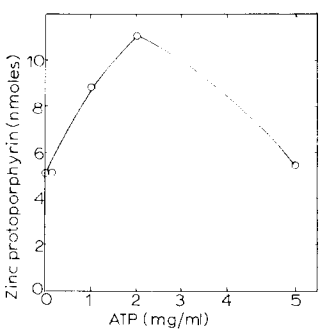
Fig. 5. Activation of ferrochelatase activity by glutathione. Ferrochelatase activity is shown for enzyme preincubated with 5.3 mM glutathione for various periods of time. The enzyme, dissolved in Tris-HCl buffer (0.1 M, pH 8.0), was preincubated in open tubes at 25° for the period indicated and then assayed, after addition of porphyrin and iron, for ferrochelatase activity.

Fig. 6. Inhibition of ferrochelatase activity by iodoacetamide and *p*-chloromercuribenzoate. The enzyme was preincubated at 25° in Tris–HCl buffer (0.1 M, pH 8.0) containing 0.6 mM dithiothreitol for 20 min. The indicated amounts of inhibitor were then added and the preincubation continued for an additional 20 min. Mesoporphyrin and iron were added and the mixture assayed for ferrochelatase activity by the standard procedure. ●—●, *p*-chloromercuribenzoate; ○—○, iodoacetamide.

Effect of other activators and inhibitors

The activation of ferrochelatase activity by sulfhydryl-reducing agents suggests that sulfhydryl groups may be involved in the action of metal chelatases. While zinc-chelatase activity was not appreciably affected by either *p*-chloromercuribenzoate or iodoacetamide, ferrochelatase was markedly inhibited. As indicated in Fig. 6 these two reagents caused maximum inhibition of 62 and 93%, respectively.

ATP consistently stimulated zinc-chelatase activity 1.5–2.5-fold. Fig. 7 indicates that maximal stimulation occurred at about 2.5 mg ATP per ml of reaction mixture or at a molar ratio of ATP to zinc of about 50:1. The relatively high concentration necessary for stimulation is difficult to explain. Loss of ATP by enzymatic hydrolysis would not seem to be a major factor since separate experiments have shown that less than 5% of the ATP would have been hydrolyzed under the assay conditions employed. In contrast to its effect on zinc-chelatase activity, ATP is strongly inhibitory for ferrochelatase. Concentrations of 0.1 and 2.0 mg/ml inhibited ferrochelatase 30 and 67%, respectively.



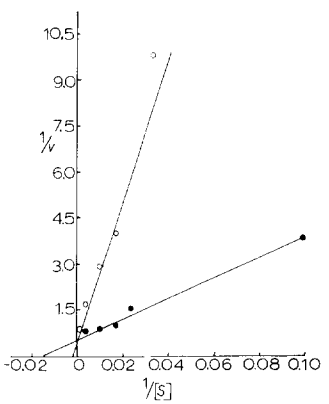


Fig. 7. Effect of ATP on zinc-chelatase activity. Enzyme activity expressed as nmoles of zinc protoporphyrin formed in 4 min, was determined by the standard fluorimetric assay except that the concentration of ATP was varied as indicated.

Fig. 8. Lineweaver–Burk plot of the effect of Fe^{2+} on zinc-chelatase activity. Zinc-chelatase activity was observed by the standard fluorimetric assay except that the concentration of zinc was varied as indicated. In addition to the normal components, the reaction mixture contained 20 nmoles of ascorbic acid. Substrate concentration [S] is expressed as μM zinc, and velocity (v) is expressed as nmoles of zinc protoporphyrin formed per min. ●—●, no inhibitor; ○—○, 1 mM Fe^{2+}.

Both Fe^{2+} and Co^{2+} inhibited zinc-chelatase activity. The data in Fig. 8 indicate that the inhibition by Fe^{2+} was competitive, and that the apparent K_i was 0.14 mM. In this experiment ascorbic acid (5 mM) was used to maintain the iron in the ferrous state. 1.0 mM Fe^{3+} was not found to inhibit enzyme activity when zinc chelatase was assayed by the standard spectrophotometric procedure. Zinc-chelatase activity, when assayed fluorimetrically, in the presence of 1.0 mM Co^{2+}, was about 50% inhibited, and this inhibition appeared to be at least partially competitive.

In view of previous work[7,22] which showed marked stimulation by various organic solvents and lipids of a metal chelatase from *R. spheroides*, the effect of some of these reagents on the barley enzyme was tested. The standard spectrophotometric

assay for zinc-chelatase activity was employed except that 0.1 ml of organic solvent was added to the incubation mixture before incubation. Glycerol showed a 1.5-fold stimulation, and acetone had no effect, but ethyl ether, ethyl acetate, and ethyl methyl ketone caused marked inhibition.

DISCUSSION

Zinc-chelatase activity has been previously measured by uptake of ^{65}Zn (see refs. 4 and 7), by spectrophotometric determination of zinc-protoporphyrin formation[7] and by disappearance of band IV of the porphyrin spectrum[23]. The basis of the fluorimetric assay described in the present report is the increase in fluorescence due to zinc-protoporphyrin formation. Because the preparation of the sample for assay is uncomplicated and because of the sensitivity of fluorescence measurement, the procedure described provides a rapid and sensitive assay for zinc-chelatase activity. Reaction time for this assay is one fifth or less that required for previously used procedures.

Ferrochelatase from plant tissue, as well as that from animal and microbial tissue has generally been reported to occur associated with the particulate fraction[14]. Possibly because of this, few attempts have been made to fractionate this enzyme extensively. RIETHMUELLER AND TUPPY[6] have, however, reported a 75-fold purification of ferrochelatase from yeast mitochondria. On the other hand, the enzyme that we have studied has been partially extracted from etiolated barley leaves in a soluble form without the aid of detergents. This soluble enzyme has been purified about 30-fold.

Conclusive evidence that a single enzyme is responsible for the insertion of both zinc and iron into porphyrins will have to await further purification of the enzyme; however, the following considerations lend some support to such a view. Zinc- and ferrochelatase activity are fractionated, both qualitatively and quantitatively, in a very comparable manner through a number of purification operations. Also, in this connection, we have shown that the elution patterns of the two activities from Sephadex G-150 are very similar. The common pH optimum at pH 8.1 also suggests that a single enzyme is involved. Likewise, the kinetic data which indicate that the inhibition of zinc chelatase by Fe^{2+} is competitive strongly suggest that both zinc and iron compete for a common site on the enzyme.

On the other hand, observations concerning the activation and inhibition of the metal chelatase suggest that two types of activity are concerned with the enzymatic chelation of zinc and iron by porphyrins. While ATP clearly stimulates zinc-chelatase activity, it has a strongly inhibitory effect on ferrochelatase activity. The explanation for the stimulation of zinc insertion is not known but it may be related to the established ability of adenosine-5'-phosphate to form complexes with metals[24]. Possibly zinc–ATP, either free or bound to the enzyme in a ternary complex, serves as a more effective metal donor to form zinc protoporphyrin than does uncomplexed zinc. PHILLIPS[25] has reported, for example, that the 1:1 copper–8-hydroxyquinoline complex, apparently by affecting the entropy of activation, greatly stimulated the rate of incorporation of copper ion from aqueous detergent solution with the porphyrin nucleus.

A distinction between zinc- and ferrochelatase activity is also evident in the

effect that sulfhydryl-reducing reagents show toward the two activities. The stimulation of ferrochelatase activity by glutathione and dithiothreitol was not observed for zinc-chelatase activity. The enhancement of ferrochelatase activity may be due to more than one cause. Since Fe^{3+} clearly does not serve as a substrate and since Fe^{2+} would be oxidized under the experimental conditions employed[13], it is clear that the reducing agents function, in part at least, to maintain the presence of Fe^{2+}. Although JONES[13] observed that 250 mM mercaptoethanol was required to remove all of the dissolved O_2 in aqueous medium, we have observed maximal stimulation of ferrochelatase after aerobic preincubation with 0.5 mM dithiothreitol and 5.0 mM glutathione. This observation, coupled with the fact that the degree of stimulation observed could be related to the period of incubation of the enzyme with reducing agent in the absence of Fe^{3+}, would indicate that some effect other than on the oxidation level of the iron may be involved. The strong inhibition of ferrochelatase activity by the sulfhydryl inhibitors, *p*-chloromercuribenzoate and iodoacetamide is also evidence that one or more sulfhydryl groups may be involved in ferrochelatase activity. No such evidence was obtained concerning zinc-chelatase activity.

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