BBA 65848

THE METALLOPROTEIN NATURE OF USTILAGO δ-AMINOLEVULINATE DEHYDRATASE*

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(Received August 13th, 1968)

SUMMARY

1. The δ-aminolevulinate dehydratase of *Ustilago sphaerogena* has been purified approx. 40-fold.

2. The enzyme was strongly inhibited by a number of nonspecific metal-binding agents, such as 1,10-phenanthroline. The inhibitory power of the ligands was greatly augmented by preincubation with the enzyme.

3. Agents specific for Cu^{+} were powerful inhibitors, while those specific for Cu^{2+} were without effect.

4. Attempts to reconstitute the ligand-treated enzyme by incubation with various copper compounds were frustrated by the extreme susceptibility of the enzyme to inhibition by copper.

5. Partial reactivation of the ligand-treated enzyme could be achieved with Zn^{2+}.

6. A moderately good correlation was found between enzyme activity and the copper content of the purified enzyme.

INTRODUCTION

The smut fungus, *Ustilago sphaerogena*, when grown in its sporidial stage in synthetic media containing certain nutrients, is capable of cytochrome *c* synthesis to the extent of one percent of the dry weight of the cells[1]. *U. sphaerogena* is hence a suitable organism in which to examine the various factors regulating the activity of the series of enzymes engaged in the biosynthesis of porphyrin.

We showed previously that the potency of δ-aminolevulinate dehydratase (EC 4.2.1.24) in crude extracts of *U. sphaerogena* cells is dependent on the Zn^{2+} concentration of the media and it was demonstrated that zinc is involved in the biosynthesis of the enzyme[2].

The condensation of two moles of δ-aminolevulinate to form porphobilinogen consists of aldol condensation, Schiff base formation and dehydration. As with

Abbreviations: BCS, sodium bathocuproinedisulfonate (2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonate).

* Abstracted from the doctoral dissertation of Hirochika Komai, University of California, Berkeley, 1968.

enzyme-catalyzed aldol condensation[3,4] and Schiff base formation[5], the reaction promoted by δ-aminolevulinate dehydratase involves an enzyme–substrate Schiff base intermediate[6]. In addition, SH group(s) appears to participate in the reaction[7–10]. Metal ions have been implicated with the enzyme from certain sources[8,9]; however, in no instance has the enzyme thus far been demonstrated conclusively to be a metalloprotein.

In the present communication we report purification of δ-aminolevulinate dehydratase from *U. sphaerogena* and present evidence that a tightly bound metal ion, possibly Cu^+, is essential for the activity of the enzyme.

MATERIALS AND METHODS

With the exception of δ-aminolevulinic acid hydrochloride, which was prepared according to the method of NEUBERGER AND SCOTT[11], all chemicals used were reagent-grade commercial products. Distilled water was used throughout. A Beckman DU-Gilford apparatus was used for all spectrophotometric assays, and a Bausch and Lomb Spectronic instrument was used for the assay of protein. Atomic absorption analyses were performed with a Perkin–Elmer model 303 apparatus equipped with a null recorder readout attachment.

The media employed for the culture of *U. sphaerogena* were similar to those used previously[12] and contained the following ingredients per l: cane sugar, 20 g; monobasic potassium phosphate, 3 g; ammonium acetate, 3 g; K_2SO_4, 1 g; citric acid, 1 g; thiamine chloride hydrochloride, 2 mg; Mg^{2+}, 80 mg; Fe^{2+}, 16 mg; Zn^{2+}, 2 mg; Mn^{2+}, 0.035 mg; Cu^{2+}, 0.005 mg. The pH of the medium was adjusted to 6.8 with concentrated NH_4OH prior to sterilization by autoclaving. Inocula (2%, v/v) grown 2 days in the same medium were used routinely, and cells were cultured aerobically at 30° for 3 days.

The activity of δ-aminolevulinate dehydratase was assayed essentially by the method of GIBSON *et al.*[10]. The assay mixture contained potassium phosphate buffer (pH 7.0) 0.3 mmole; δ-aminolevulinic acid hydrochloride, 10 μmoles; enzyme and any additional ingredients, *plus* distilled water, to a final vol. of 3.0 ml. Unless otherwise noted, incubations were carried out in evacuated Thunberg tubes at 37°. A unit of enzyme was defined as that amount which produced 1 nmole of product per h, using 34.6 as the millimolar extinction coefficient of porphobilinogen at 555 mμ (ref. 13).

Protein was determined according to the procedure of LOWRY *et al.*[14], using bovine serum albumin (Armour) as a standard. In the case of crude extracts, protein was measured on a 5% trichloroacetic acid precipitate redissolved in 1 M NaOH.

RESULTS

Extraction and purification of the enzyme

All steps were carried out at 5°. Unless otherwise specified, the buffer contained 0.01 M β-mercaptoethanol and was comprised of phosphate solution (pH 7.0) at an indicated molarity.

The cells were collected by centrifugation, ground with alumina, and 250 ml of 0.1 M buffer added per cell mass derived from each 5 l of culture. After centrifugation at 30 000 × *g* for 30 min, the supernatant was filtered through glass wool to remove

floating lipids. After the addition of 24.5 g $(NH_4)_2SO_4$ per 100 ml crude extract, the preparation was centrifuged at 17 000 × *g* for 10 min, an additional 10.5 g $(NH_4)_2SO_4$ was added to the supernatant, the precipitate collected by centrifugation and dissolved in a vol. of 0.1 M buffer equal to one-fifth that of the crude extract. The preparation was then heated in a water bath at 55° for precisely 5 min, cooled, centrifuged and stored in the frozen state prior to combination with similar batches destined for further purification.

The supernatant solutions from the heat step were thawed, combined, and a second $(NH_4)_2SO_4$ fractionation was carried out as described above. The precipitate was dissolved in a volume of 0.01 M buffer equal to 1/100 of that of the crude extract, dialyzed overnight against 0.01 M buffer and applied to a column of DEAE-cellulose previously equilibrated with the same buffer. A column 2.2 cm × 20 cm in size was used for the enzyme from 5 l of medium. After thorough washing of the column with buffer, the enzyme was eluted with a linear gradient prepared from 0.01 M buffer (mixing chamber) and 0.01 M buffer–0.5 M NaCl (reservoir). The active fractions were pooled, $(NH_4)_2SO_4$ was added to a level of 35 g/100 ml, the precipitate was collected by centrifugation and dissolved in a volume of 0.01 M buffer equal to 1/300 of that of the crude extract. The preparation was then filtered through Sephadex G-200 using 0.01 M buffer as solvent and a column size of 1 cm × 100 cm for enzyme from 5 l of medium. The V_e/V_0* found for the single peak with enzyme activity (1.4) was just slightly less than that measured for catalase on the same column. This suggests that the molecular weight of the enzyme is of the order of $2 \cdot 10^5$–$3 \cdot 10^5$.

Batch adsorption on calcium phosphate gel was then accomplished by using 15 mg of gel for the enzyme from each 5 l of culture medium. The gel was washed successively with 0.01 M and 0.025 M buffer and the enzyme eluted with 0.2 M buffer, using several 0.5-ml aliquots per 15 mg gel.

Table I gives a summary of the purification procedure. Even the purest preparation was found by disc electrophoresis at pH 9.5 to contain several protein components. Incubation of the gels in the assay mixture for 1 h at 37°, followed by immersion in Ehrlich reagent, revealed only a single pink band with a migration distance about one-third that of the tracing dye. The enzyme, which had a light tan color, was kept stored in the frozen state. Purified preparations required activation

TABLE I

SUMMARY OF PURIFICATION PROCESS FOR PREPARATION No. 138 OF USTILAGO δ-AMINOLEVULINATE DEHYDRATASE

Purification stage	*Vol. (ml)*	*Protein (mg)*	*Specific activity (units/mg)*	*Yield (%)*
1. Crude extract of cells from 25 l culture	1310	13 000	28	(100)
2. First $(NH_4)_2SO_4$ precipitate	262	1 900	90	63
3. Heated supernatant	262	1 580	98	42
4. Second $(NH_4)_2SO_4$ precipitate	21.8	462	228	29
5. DEAE-cellulose eluate	114	165	534	24
6. Sephadex G-200 eluate	15.7	50	1240	17
7. $Ca_3(PO_4)_2$ eluate	4.1	17	1360	6.3

* V_e, elution volume; V_0, void volume.

by a thiol compound and prior to assay the enzyme was exposed for at least 30 min to 0.01 M β-mercaptoethanol.

Inhibition by metal-binding agents

Table II lists the inhibitory power of various ligands which were added at time zero, and which were thus present during the activity assay. The addition of 1 equiv of iron ($0.33 \cdot 10^{-3}$ M) just prior to insertion of the enzyme completely eliminated the inhibition of 10^{-3} M 1,10-phenanthroline. By use of suitable controls it was shown that none of the reagents used in these and in subsequent similar experiments interfered with the assay of porphobilinogen.

TABLE II

INHIBITION OF USTILAGO δ-AMINOLEVULINATE DEHYDRATASE BY LIGANDS PRESENT DURING THE ACTIVITY ASSAY

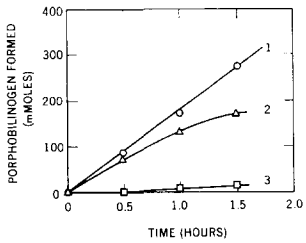
Data shown are for a dialyzed crude extract. When 8-hydroxyquinoline was added the values are corrected for the effect of the ethanol in which the ligand was dissolved. When KCN was added incubations were carried out in closed but not evacuated Thunberg tubes in order to avoid possible loss of HCN.

Added ligand	*Concn. (mM)*	*Inhibition (%)*
1,10-Phenanthroline	1.0	89
	5.0	96
2,2-Bipyridine	1.0	26
	5.0	64
8-Hydroxyquinoline	1.0	15
	5.0	61
Ethylenediaminetetraacetate	1.0	21
	5.0	50
KCN	1.0	nil
	5.0	51
Ethylenediamine di (*o*-hydroxyphenylacetate) ("Chel 138")	5.0	5
NaN_3	5.0	nil
NaF	5.0	nil

The effect of both incubation and preincubation of the enzyme with 1,10-phenanthroline is shown in Fig. 1. This inhibition is not instantaneous but is progressive with time. For example, with an enzyme preparation which was 48% inhibited by $5 \cdot 10^{-4}$ M 1,10-phenanthroline added at time zero, preincubation with the same concentration of inhibitor for 0.5, 1.0 and 2.0 min gave 64, 70 and 76% inhibition, respectively.

Inhibition by copper-binding agents

The ligands listed in Table II are nonspecific in that they combine with a variety of metal ions. In an attempt to define the nature of the metal ion in the enzyme, experiments were performed with the Cu^+-specific agents: sodium bathocuproinedisulfonate (BCS; 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonate) and neo-



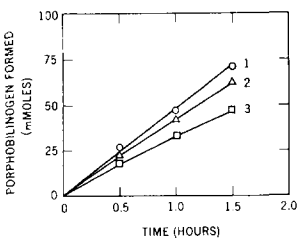


Fig. 1. Inhibition of Ustilago δ-aminolevulinate dehydratase by incubation and preincubation with 1,10-phenanthroline. Enzyme preparation used was a crude extract. Curve 1, no 1,10-phenanthroline (control) was added; Curve 2 shows the effect of direct addition to the activity assay of 1,10-phenanthroline $5 \cdot 10^{-4}$ M; Curve 3, the effect of allowing the same concentration of inhibitor to preincubate with the enzyme at room temperature for 30 min prior to addition of substrate. The standard assay conditions were employed.

Fig. 2. Inhibition of Ustilago δ-aminolevulinate dehydratase by incubation and preincubation with sodium bathocuproinedisulfonate. Enzyme preparation No. 140 (second $(NH_4)_2SO_4$ precipitate, 108 units /mg) was incubated for 30 min at room temperature with buffer (Curve 1, control) or $5 \cdot 10^{-5}$ M BCS (Curve 3). After the 30-min period, $5 \cdot 10^{-5}$ M sodium bathocuproinedisulfonate was added to the tube represented by Curve 2, and the reaction started by the addition of substrate to all tubes. The standard assay conditions were employed.

cuproine (2,9-dimethyl-1,10-phenanthroline), the Cu^{2+}-specific agent, cuprizone (*bis*-cyclohexanoneoxaldihydrazone), and diethyldithiocarbamate. As seen in Fig. 2, the presence of $5 \cdot 10^{-5}$ M BCS during the reaction gave significant inhibition, about 14%, while preincubation with the ligand, as with 1,10-phenanthroline (see Fig. 1), gave much higher levels of inhibition. Similar experiments with cuprizone were complicated by the necessity of dissolving the reagent in ethanol. Also, in this instance the $CuSO_4$ treatment for elimination of thiol compounds had to be omitted from the assay procedure for porphobilinogen. In spite of these limitations, it was concluded that 10^{-4} M cuprizone gave no detectable inhibition with or without preincubation. Diethyldithiocarbamate was similarly ineffective as an inhibitor. The data summarized in Table III show that inhibitory power is confined to the Cu^{+}-specific ligands.

TABLE III

INHIBITION OF USTILAGO δ-AMINOLEVULINATE DEHYDRATASE BY INCUBATION AND PREINCUBATION WITH COPPER-BINDING AGENTS

Data shown are for enzyme preparations purified through the second $(NH_4)_2SO_4$ precipitation stage and containing approx. 150 units/mg. Cuprizone was dissolved in warm ethanol.

	Inhibition (%)				
	Direct addition to activity assay			*Preincubation 30 min at room temp.*	
Ligand concn. (M):	$1 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	$5 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$5 \cdot 10^{-5}$
Bathocuproinedisulfonate (K and K Laboratories)	59	—	14	55	36
Neocuproine (Matheson Coleman and Bell)	—	18	—	56	—
Cuprizone (G. Frederick Smith Co.)	—	nil	—	nil	—
Diethyldithiocarbamate (Matheson Coleman and Bell)	nil	—	—	nil	—

TABLE IV

EFFECT OF COPPER COMPOUNDS ON UNTREATED AND BCS-TREATED USTILAGO δ-AMINOLEVULINATE DEHYDRATASE

Data shown are for enzyme preparation No. 141 purified through the second $(NH_4)_2SO_4$ precipitation stage.

Copper compounds	*Conditions*	*Specific activity (units/mg)*
CuCl; $CuCl_2$	Control (untreated enzyme preincubated 1 h at room temp. in buffer)	183
	After preincubation 1 h at room temp. with $1 \cdot 10^{-3}$ M BCS	43
	After BCS treatment and exhaustive dialysis	83
	After BCS treatment, dialysis and preincubation for 2 h at room temp. with $1 \cdot 10^{-5}$ M CuCl*	27
	After BCS treatment, dialysis and preincubation for 5 h at 5° with $5 \cdot 10^{-5}$ M $CuCl_2$	20
$Cu(CH_3CN)_4ClO_4$	Control (untreated enzyme)	183
	Direct addition to the activity assay of $Cu(CH_3CN)_4ClO_4$ at the following concentrations:	
	$1 \cdot 10^{-4}$ M	16
	$1 \cdot 10^{-5}$ M	49
	$1 \cdot 10^{-6}$ M	65
	$1 \cdot 10^{-7}$ M	162
	Control (untreated enzyme preincubated 1 h at room temp. in buffer)	183
	After preincubation 1 h at room temp. with $1 \cdot 10^{-3}$ M BCS	37
	After BCS treatment and exhaustive dialysis	53
	After BCS treatment, dialysis and direct addition of $Cu(CH_3CN)_4ClO_4$ to the activity assay at the following concentrations:**	
	$1.7 \cdot 10^{-8}$ M	55
	$3.3 \cdot 10^{-8}$ M	51
	$8.0 \cdot 10^{-8}$ M	57
	$1.6 \cdot 10^{-7}$ M	45
	After BCS treatment, dialysis and preincubation for 2 h at room temp. with $Cu(CH_3CN)_4ClO_4$ at the following concentrations:	
	$1 \cdot 10^{-8}$ M	53
	$5 \cdot 10^{-8}$ M	53
	$1 \cdot 10^{-7}$ M	54
	$2.5 \cdot 10^{-7}$ M	46
$CuSO_4$	Control (untreated enzyme preincubated 1 h at room temp. in buffer)	183
	After preincubation 1 h at room temp. with $CuSO_4$ at the following concentrations:	
	$1 \cdot 10^{-3}$ M	18
	$1 \cdot 10^{-4}$ M	22
	$1 \cdot 10^{-5}$ M	64
	$1 \cdot 10^{-6}$ M	109
	Direct addition of $1 \cdot 10^{-5}$ M $CuSO_4$ to the activity assay without preincubation	129

* Reagent dissolved in 0.1 M phosphate buffer (pH 7) containing $1 \cdot 10^{-2}$ M β-mercaptoethanol.

** Reagent ($1 \cdot 10^{-3}$ M) was dissolved in acetonitrile.

Attempts to reconstitute the BCS-treated enzyme and inhibition by copper

A number of experiments were carried out in an attempt to restore enzyme activity following a 1 h exposure to 10^{-3} M BCS. Aliquots of enzyme containing 0.34 mg protein per ml were used throughout and the buffer, as always, contained 0.01 M β-mercaptoethanol. Dialysis in the cold for 24 h against three 1-l changes of buffer was used to remove excess BCS, and this led to some restoration of activity. The untreated enzyme upon similar dialysis did not change activity.

Unfortunately, as illustrated in Table IV, the addition of either CuCl or $CuCl_2$ at levels of about 10^{-5} M still further reduced the activity of the BCS-treated enzyme. Even cuprous acetonitrile perchlorate, the reagent of choice for reconstitution of copper proteins[15,16], began to display inhibitory effects at levels of about 10^{-7} M. Finally, as shown in Table IV, a test with various concentrations of $CuSO_4$ revealed the potent inhibitory effect of copper. To a considerable degree this inhibition is prevented by the substrate.

Partial restoration of activity of the BCS-treated enzyme with Fe^{2+} and Zn^{2+}

When metal ions other than Cu^{2+} were examined, it was consistently found that Zn^{2+}, and to a barely detectable extent, Fe^{2+} could partially restore activity of

TABLE V

EFFECT OF Zn^{2+} ON BCS-TREATED USTILAGO δ-AMINOLEVULINATE DEHYDRATASE

Data shown are for enzyme preparation No. 140 purified through the second $(NH_4)_2SO_4$ precipitation stage.

Conditions	*Specific activity (units/mg)*
Control (untreated enzyme preincubated 1 h at room temp in buffer)	108
After preincubation 1 h at room temp. with $1 \cdot 10^{-3}$ M BCS	26
After BCS treatment and exhaustive dialysis	48
After BCS treatment, dialysis and preincubation 2 h at room temp. with $1 \cdot 10^{-5}$ M $ZnCl_2$	61

the BCS-treated enzyme (Table V). Other bivalent metal ions such as Mn^{2+}, Co^{2+} and Ni^{2+} were inactive. Zn^{2+} at a concentration of 10^{-5} M had a negligible effect on the untreated enzyme either before of after exhaustive dialysis.

Correlation of enzyme activity and metal ion content

Atomic absorption analysis was applied to highly purified preparations of the enzyme in order to determine the concentration therein of various metal ions. Table VI shows that significant amounts of Fe^{2+}, Zn^{2+} and Cu^{2+} were present; of these ions only the latter followed the enrichment of activity. Denatured enzyme may have been present in the $Ca_3(PO_4)_2$ eluate in view of the low yield of activity in this step (see Table I). Co^{2+} and Mn^{2+} were below the limits of quantitative determination.

Three purified preparations were subjected to rechromatography on DEAE-cellulose so as to provide a series of fractions of varying dehydratase potency, each of which could be analyzed for both enzyme and metal ion content. The results, as recorded in Table VII, are somewhat ambiguous in that none of the three metal ions

TABLE VI

METAL ION CONTENT OF PREPARATION No. 138 OF USTILAGO δ-AMINOLEVULINATE DEHYDRATASE AT VARIOUS STAGES OF PURIFICATION

Purification stage	*Specific activity (units/mg)*	*Metal/protein (ng atoms/mg)*		
		Fe^{2+}	Zn^{2+}	Cu^{2+}
DEAE eluate	534	4.0	2.2	0.5
Sephadex G-200 eluate	1240	1.6	2.2	0.9
$Ca_3(PO_4)_2$ eluate	1360	4.2	1.9	3.1

displayed perfect synchrony with the enzyme. However, Cu^{2+} was again the only element occurring in concentrations which were in reasonable agreement with enzyme activity.

Increasing the Cu^{2+} concentration of the growth medium 100-fold did not measurably affect the specific activity of the crude extract.

TABLE VII

CORRELATION OF ENZYME ACTIVITY AND METAL ION CONTENT OF DEAE-CELLULOSE CHROMATOGRAPHIC FRACTIONS OF THREE PREPARATIONS OF USTILAGO δ-AMINOLEVULINATE DEHYDRATASE

Preparation No.	*Fraction No.*	*Activity (units/ml)*	*Metal ion concn. (ng atoms/ml)*		
			Fe^{2+}	Zn^{2+}	Cu^{2+}
125	6	80	1.1	1.5	1.0
	7	566	2.6	1.5	0.8
	8	388	5.0	1.8	0.9
	9	152	6.3	1.6	0.6
126	4	272	0.8	1.5	1.6
	5	850	1.6	1.7	1.4
	6	794	2.8	1.7	1.4
	7	616	3.5	2.8	1.2
	8	250	4.7	2.5	1.0
	9	136	6.5	1.3	0.6
128	5	20	1.3	2.2	1.6
	6	1220	1.9	4.1	1.7
	7	1230	3.8	2.4	1.7
	8	658	5.0	2.8	1.4
	9	242	7.2	2.2	1.3
	10	114	8.5	2.1	1.0
	11	46	8.9	1.6	1.1

DISCUSSION

Like δ-aminolevulinate dehydratase from other sources[7–10], the Ustilago enzyme requires essential thiol groups for activity. The enzyme could be poisoned by iodoacetate, *p*-chloromercuribenzoate, Cu^{2+} and by high concentrations of Zn^{2+}. Although the crude extract could be dialyzed with impunity against cold 0.01 M phos-

phate buffer, the addition of 0.01 M β-mercaptoethanol was required at later stages in order to activate the enzyme.

With the exception of the enzyme from wheat leaves, which is activated by metallic cations[8], unequivocal evidence has thus far been lacking as to the involvement of metal ion(s) in the reaction catalyzed by δ-aminolevulinate dehydratase. GRANICK AND MAUZERALL[9] reported inhibition of the avian red blood cell enzyme with amine-acetate chelating agents; other complexing substances were not tried. GIBSON *et al.*[10] found 8-hydroxyquinoline-5-sulfonate and 8-hydroxy-7-iodoquinoline-5-sulfonate to inhibit the ox liver enzyme. However, these ligands also inhibited in the presence of excess Fe^{3+} and the authors concluded the effect might have been due to the sulfonate group.

The experiments reported in the present investigation disclose the probable metalloprotein nature of Ustilago δ-aminolevulinate dehydratase. In view of the substantial inhibition noted with ligands of quite different structure, it is unlikely that these reagents act by a mechanism other than metal binding. The varying degree of inhibition observed with ligands of comparable affinity for metal ions suggests an initial combination of reagent with the protein-bound metal ion. The thermodynamics and kinetics of such a process would be governed by the structures of the ligands and the enzyme, *i.e.*, factors which would be largely irrelevant if the inhibitor were merely capturing a free metal ion dissociated from the enzyme by the equilibrium:

$$\text{Enzyme-bound metal ion} \rightleftharpoons \text{apoenzyme} + \text{free metal ion}$$

Our observations are compatible with the postulate that an inactive enzyme (E_0') is formed slowly from the enzyme–inhibitor complex (EI) which is in rapid equilibrium with the free enzyme (E) and inhibitor (I).

$$E + I \rightleftharpoons EI \xrightarrow{\text{slow}} E'$$

If there were only an enzyme–inhibitor complex in rapid equilibrium with free enzyme and inhibitor, then there should be no increase in the extent of inhibition when the enzyme is preincubated with the ligand in the absence of the substrate.

Substrate protection against inhibition by chelating agents suggests the involvement of bound metal ion in one way or another with the active site of the enzyme. In experiments not reported in detail here, we found that the intact enzyme, in contrast to the ligand-treated enzyme, was more susceptible to inactivation with $NaBH_4$ in the presence of substrate. It has been shown that the δ-aminolevulinate dehydratase reaction proceeds by initial formation of an enzyme–Schiff base intermediate followed by aldol condensation on a second mole of substrate[6]. The catalysis by metal ions of reactions involving Schiff bases has been reported[17,18]. GRANICK AND MAUZERALL[9] speculated that a metal ion might facilitate the aldol condensation catalyzed by δ-aminolevulinate dehydratase through increasing the ability of the active methylene group to enolize.

All of the agents indicated in Table II to be effective inhibitors are also efficient copper-binding substances. The inhibition by BCS, an agent which binds neither iron nor zinc, is about as powerful as that exhibited by 1,10-phenanthroline, the latter being the most potent of the nonspecific ligands. These data, together with the

moderately good correlation of activity and copper content of the purified enzyme, would appear to implicate copper as an essential bound metal ion in Ustilago δ-aminolevulinate dehydratase. Purified liver δ-aminolevulinate dehydratase was found to contain 0.1% copper, but "essentially" all of the metal ion could be removed by dialysis against cyanide without parallel loss in activity[19]. Copper, as well as iron, has long been recognized as necessary for the biosynthesis of hemoproteins. The association of copper with δ-aminolevulinate dehydratase, which is a nonoxidative enzyme, is remarkable and needs to be confirmed by isolation and analysis of the pure enzyme and by working out conditions for removal and reinsertion of the specific metal ion. A partial substitution of zinc for copper, which may be a consequence of the similar electron structure of these elements, would rule out an internal oxidation–reduction mechanism for the metal ion in Ustilago δ-aminolevulinate dehydratase.

ACKNOWLEDGMENTS

This research was supported in part by U.S. Public Health Service Grants No. AI-04156 and AM-8845, and by National Science Foundation Grant No. GB-5276X. The authors are indebted to Dr. J. KERBER and Dr. H. SANUI for assistance with the atomic absorption analyses. Dr. H. MASON kindly provided a sample of cuprous acetonitrile perchlorate.

NOTE ADDED IN PROOF (Received November 1st, 1968)

Recently it has been discovered that beef liver δ-aminolevulinate dehydratase, purified through the first ammonium sulfate precipitation stage[10] and containing 30 units/mg, displays a pattern of inhibition with neocuproine and bathocuproine-disulfonate essentially similar to that found for the Ustilago enzyme (see Table III).

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