Biochimica et Biophysica Acta, 293 (1973) 497–505

BBA 66824

INHIBITION OF UROCANASE BY CUPRIC ION

DANIEL H. HUG AND DENNIS ROTH
Bacteriology Research Laboratory, Veterans Administration Hospital, Iowa City, Iowa (U.S.A.)

(Received September 5th, 1972)

SUMMARY

Purified photoactivated urocanase from histidine-grown *Pseudomonas putida* was activated 0–25% by histidine depending on the enzyme batch tested. EDTA could substitute for histidine. Subsequently, 11 divalent cations were tested for inhibition of urocanase. The enzyme was sensitive to Cu^{2+} (50% inhibition at 0.3 μM). This inhibition was not enhanced by preincubation of enzyme for 15 min with Cu^{2+}. A double-reciprocal plot of activity *vs* substrate concentration suggested that the inhibition by Cu^{2+} was noncompetitive. Inhibition was prevented completely if a metal-binding agent was added prior to the substrate. If the metal-binding agent was added after the substrate, the activity was partially restored. Substances that prevented the 70% inhibition by 1.67 μM Cu^{2+} were histidine (6.7 μM), EDTA (3.3 μM), thiol-histidine (3.3 μM), ergothioneine (3.3 μM), penicillamine (3.3 μM), cysteine (6.7 μM), histamine (17 μM) and albumin (66 μg/ml). Cu^{2+} had no effect on photoactivation of inactive urocanase. The behavior of purified native urocanase with respect to inhibition by Cu^{2+} was similar to the photoactivated enzyme.

INTRODUCTION

The photoactivation of urocanase from *Pseudomonas putida* has been described in reports from our laboratory[1,2]. In studying metabolites as possible effectors of urocanase and their relation to the suggested photoregulation of the histidine-degrading pathway[3], we found that some batches of purified photoactivated urocanase were activated by L-histidine. EDTA was able to substitute for histidine in this activation. These findings led to the experiments reported here which indicated that urocanase is inhibited by cupric ions at low concentrations.

MATERIALS AND METHODS

General

The growth of *P. putida* A.3.12 on a medium containing L-histidine and yeast extract, storage of cells, their disruption by ultrasound and purification of inactive urocanase have been described[1,2]. Active urocanase (native enzyme) was prepared from freshly grown cells by the same purification procedure[2].

Urocanase assay

The enzyme was assayed by recording the change in absorbance of urocanate at 277 nm as previously described[2]. The cuvette contained 0.05 ml of purified inactive urocanase (Fraction 5, 6 μg protein)[2] in a total volume of 3.0 ml. The Beckman Model DU spectrophotometer connected to a Beckman 10 inch recorder was used. A unit was defined as the enzyme activity which catalyzed the reaction of 1 μmole of substrate per min.

Irradiation

Irradiation by near-ultraviolet light photoactivates inactive urocanase. Two 15 W General Electric black light lamps (distance of 10 cm; 15 min; filtered, peak transmittance at 356 nm) were utilized as previously described[1] except the samples were at room temperature.

Reagents

Divalent metals and Ag^+ were purchased from Fisher as chloride salts, certified grade, except $SnCl_2$ and $CaCl_2$ from J. T. Baker and $FeCl_2$ from Baker and Adamson. The copper salt contained 0.01% Fe and the iron salt contained 0.005% Cu by the manufacturer's analysis. Calbiochem was the source of L-carnosine, glutamine, histamine, L-histidine, D-penicillamine, L-threonine and urocanic acid. EDTA was purchased from J. T. Baker, L-cysteine from Eastman, L-thiolhistidine and L-ergothioneine from Mann and bovine serum albumin from Armour. Potassium phosphate salts were reagent grade. Materials used in the enzyme purification were previously described[2]. All solutions except media were prepared with water which had been distilled and deionized.

RESULTS

The activity of various batches of purified photoactivated urocanase, second enzyme of the histidine-degrading pathway, was increased from 0–25% by addition of 0.3 mM L-histidine. Such experiments were run 10 times in 3 months on three batches and activation of 5–25% was observed on seven occasions. Since histidine binds divalent cations, we suspected this activation might be due to metal binding. Subsequently, it was found that 0.067 mM EDTA (or less) also had the ability to activate urocanase from 5–26% on five of seven tests. Consequently we studied the effect of added metals on enzyme activity.

Effect of divalent cations on urocanase

Divalent cations were added to purified photoactivated urocanase (Table I). Of the cations surveyed at 333 μM, only Cu^{2+}, Hg^{2+} and Fe^{2+} were inhibitory. Pb^{2+} was not inhibitory in phosphate or Tris but both buffers may alter its effective concentration. The inhibition by Hg^{2+} was not studied further but it was evident that Hg^{2+} contamination of the $CuCl_2$ reagent could not account for the Cu^{2+} inhibition. The inhibition by Fe^{2+} was reproducible and the extent of inhibition was dependent on Fe^{2+} concentration. However, since about 1000 times more Fe^{2+} than Cu^{2+} was required for 50% inhibition, Fe^{2+} was not studied further. Fe^{2+} inhibition could not be accounted for by Cu impurities. There was no inhibition when 333 μM Ag^+ was tried.

TABLE I

RELATIVE ACTIVITY OF UROCANASE IN THE PRESENCE OF DIVALENT CATIONS

The assay for urocanase was described in the text. Enzyme and buffer were irradiated by long ultraviolet light for 15 min before substrate and cation were added. The concentration of added salt was 333 μM.

Addition	*Relative activity*	*Addition*	*Relative activity*
None	100	$CdCl_2$	100
$CuCl_2$	7	$CaCl_2$	100
$HgCl_2$	23	$NiCl_2$	100
$FeCl_2$	43	$ZnCl_2$	100
$SnCl_2$	89	$MgCl_2$	100
$CoCl_2$	93	$PbCl_2$*	100

* Since $Pb_3(PO_4)_2$ is insoluble, we substituted Tris buffer, pH 7.5.

The effect of Cu^{2+} concentration on the inhibition of urocanase is presented in Fig. 1. Approximately 0.3 μM Cu^{2+} inhibited urocanase by 50%. For many of the subsequent experiments, we selected 1.67 μM Cu^{2+} to give a substantial (but not maximum) inhibition. This concentration of Cu^{2+} gave, for various batches and days, an average inhibition of 69% with a range from 65–74% (see Table III).

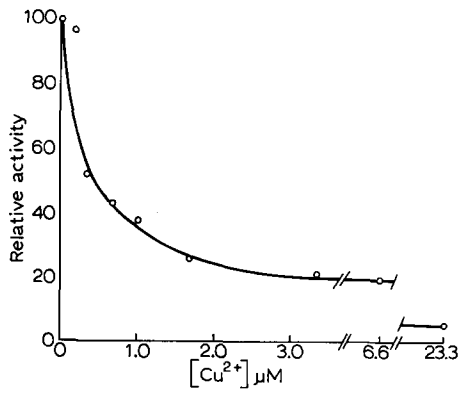

Fig. 1. Inhibition of photoactivated urocanase as a function of Cu^{2+} concentration. The enzyme was assayed as described in the text in the presence of increasing concentrations of $CuCl_2$.

Kinetics

A double-reciprocal plot of enzyme activity and substrate concentration is given in Fig. 2. The K_m for photoactivated urocanase was 0.04 mM; 0.05 mM was previously reported[2]. Whereas a decrease in maximum velocity resulted from Cu^{2+} treatment, no change in the apparent K_m for substrate was observed in the presence of Cu^{2+}. This finding suggests that Cu^{2+} inhibition might be due to formation of a catalytically inactive species of enzyme instead of a modification in the substrate-binding capacity of the enzyme.

Incubation of Cu^{2+} with urocanase

Within the limits of experimental manipulation (pipetting, mixing), the inhibi-

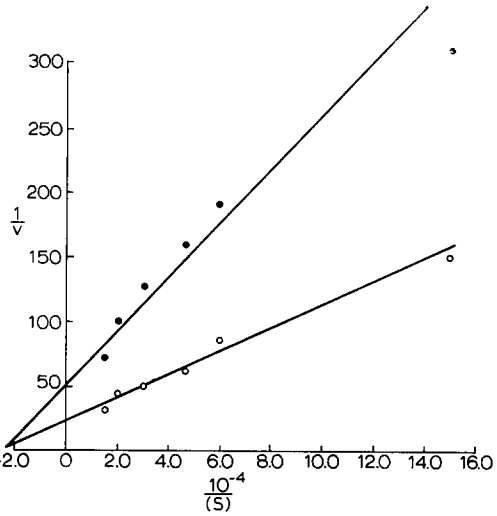

Fig. 2. Double-reciprocal plot of urocanase activity *vs* substrate concentration. Assay was performed as described in the text except substrate concentration was varied. ○, without inhibitor; ●, with inhibitor (1.67 μM $CuCl_2$). The reciprocal of the activity is in arbitrary units.

tion required no time to develop and remained constant for 15 min incubation of Cu^{2+} with the enzyme (Table II). If Cu^{2+} was acting as a catalyst for oxidation of sulfhydryl groups as in the inactivation of transglutaminase[4], we would expect a progressive loss of activity with time.

TABLE II

EFFECT OF TIME ON INHIBITION OF UROCANASE BY COPPER

The assay for purified urocanase and the photoactivation of urocanase were described in the text. The irradiated enzyme and buffer were incubated at 30 °C for the indicated time with $CuCl_2$ before the assay was started by addition of substrate.

$CuCl_2$ added (μM)	*Incubation (min)*	*Enzyme activity (munits)*
0	0	4.9
1.67	0	1.2
1.67	5	1.3
1.67	10	1.2
1.67	15	1.3

Prevention of inhibition by metal-binding agents

Various metal-binding agents, most of which were naturally occurring substances, were added to the Cu^{2+} and enzyme mixture before the addition of substrate. The concentration range effective for prevention of the inhibition by Cu^{2+} is indicated in Table III. Some metal-binding agents required only twice the Cu^{2+} concentration to completely prevent inhibition. Penicillamine is used therapeutically to bind excess Cu in Wilson's disease. Other effective agents are derivatives of histidine: ergothioneine, thiolhistidine and carnosine. Histidine, threonine and glutamine are amino acids which bind Cu^{2+} in human serum[5] especially in mixed complexes containing histidine. Thiols (as cysteine and penicillamine) are also metal-binding agents[6,7], and many of these would likely prevent Cu inhibition.

TABLE III

PREVENTION OF COPPER INHIBITION OF UROCANASE BY ADDITION OF METAL-BINDING AGENTS

The irradiation and assay of urocanase were described in the text. Before the substrate was added to start the reaction, 1.67 μM $CuCl_2$ and then the metal-binding agent were added. In the absence of $CuCl_2$, enzyme activity was taken as 100.

Metal-binding agent	*Relative enzyme activity*								
	Metal-binding agent concentration (μM)								
	0	*1.67*	*3.33*	*6.67*	*16.7*	*33.3*	*66.7*	*133*	*333*
L-2-Thiolhistidine	33	82	100						
L-Ergothioneine	32	61	100						
D-Penicillamine	30	58	100						
EDTA (Na)	34	40	100						
L-Carnosine	26		60	100					
L-Histidine·2HCl	34		64	94					
L-Cysteine·HCl	34		59	95					
Histamine·2HCl	26		60	71	100				
L-Glutamine	32					70	89		
L-Threonine	32					38	91		
Citrate, neutralized	35						56	66	74
		(μg/ml)							
	0	*33*	*66*	*133*					
Albumin, crystallized bovine serum	32	64	100	100					

TABLE IV

EFFECT OF Cu^{2+} ON PHOTOACTIVATION OF UROCANASE

Each cuvette was assayed for urocanase as described in the text. During preincubation at room temperature, some cuvettes were irradiated as described in the text for 15 min in the presence of the indicated additions. At the time of assay additions were made in the order shown in the second column. Cu^{2+} 1.67 μM; histidine 33 μM.

Conditions during preincubation	*Additions at time of assay*	*Activity (munits)*
Light	Urocanate	4.8
Light with Cu^{2+}	Urocanate	1.1
Light with Cu^{2+}	Histidine, urocanate	3.9
Light	Cu^{2+}, urocanate	1.3
Light	Cu^{2+}, histidine, urocanate	4.4
Darkness	Urocanate	0.81
Darkness	Cu^{2+}, urocanate	0.52
Darkness	Cu^{2+}, histidine, urocanate	0.74
Darkness	Histidine, urocanate	0.81

Effect of Cu^{2+} on photoactivation

It has been suggested that photoactivation involves a conformational change in urocanase[8]. If Cu^{2+} is bound to urocanase, we could ask whether it might interfere with the process of photoactivation. When Cu^{2+} was present during irradiation, inhibition was about the same as when Cu^{2+} was added after irradiation (Table IV). Prevention of inhibition by histidine was not greatly influenced whether Cu^{2+} was added before or after light treatment. If Cu^{2+} had prevented photoactivation, histidine would not be able to restore activity when added after irradiation in the

presence of Cu^{2+}. Thus, 1.67 μM Cu^{2+} inhibited urocanase but had little effect on photoactivation. Dark controls showed that Cu^{2+}, histidine or both did not activate inactive urocanase. The residual activity of inactive urocanase was also inhibited by Cu^{2+} and this inhibition was prevented by histidine.

Order of addition

The influence of the order of addition of Cu^{2+}, histidine and substrate to the enzyme-buffer solution was investigated (Table V). The order of Cu^{2+} and urocanate addition was not important (Lines 2 and 4). When histidine preceded Cu^{2+}, there was no inhibition. When histidine was added to the enzyme and Cu^{2+} mixture before urocanate (Lines 6 and 7), full activity was observed. When histidine was added to the enzyme and Cu^{2+} mixture after the substrate (Lines 3, 5), the inhibition was only partially reversed. Once the enzyme was exposed to the substrate in the presence of Cu^{2+}, a histidine concentration which was adequate to prevent inhibition did not completely restore the activity. This conclusion led us to examine the kinetics of this reversal in the next experiment.

TABLE V

EFFECT OF ORDER OF ADDITION OF Cu^{2+} AND HISTIDINE ON UROCANASE ACTIVITY

The assay for urocanase was described in the text. The enzyme and buffer were irradiated by long ultraviolet light for 15 min before substrate or other additives were present. The concentration of L-histidine was 33 μM and $CuCl_2$ was 1.67 μM when present. The substrate, Cu^{2+}, and histidine were added in the order indicated rapidly (10–15 s for each manipulation).

Additions			*Enzyme activity (munits)*
1	*2*	*3*	
Urocanate			4.7
Urocanate	Cu^{2+}		1.5
Urocanate	Cu^{2+}	Histidine	2.9
Cu^{2+}	Urocanate		1.6
Cu^{2+}	Urocanate	Histidine	2.6
Cu^{2+}	Histidine	Urocanate	4.6
Histidine	Cu^{2+}	Urocanate	4.5

In this experiment, the inhibitor and metal-binding agent were added during the course of the enzyme assay. Additions are indicated by the arrows on Fig. 3 which is a representative experiment redrawn from the tracings of the Beckman recorder. Note that when Cu^{2+} was added there was no gradual transition to a new rate (Fig. 3C) but rather the slower rate occurred at once (within the limits of the experimental manipulation). When histidine was added with the Cu^{2+} (Fig. 3D), no inhibition was observed and the relative activity was 101. When histidine was added after the Cu^{2+} (Fig. 3B), the inhibition was partly reversed (relative activity, 51) and this occurred without noticeable lag. In relative activity (Fig. 3C), the inhibited activity was 32. After reversal by histidine, the activity was 56. Glutathione (reduced), cysteine and dithiothreitol acted much like histidine in Fig. 3 (data not given); they too only partially restored activity after the substrate was present.

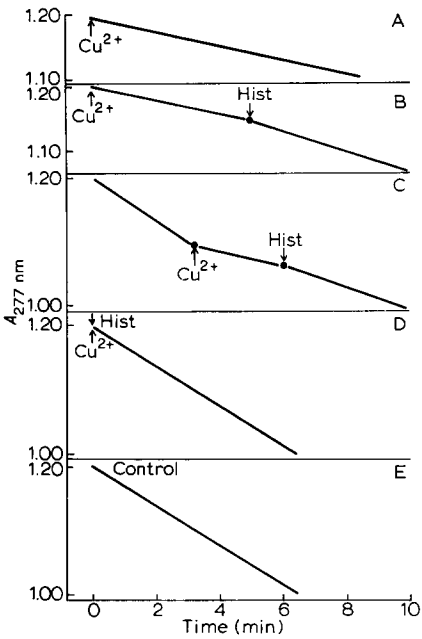

Fig. 3. The effect of histidine on photoactivated urocanase activity inhibited by Cu^{2+}. L-Histidine (33 μM) and $CuCl_2$ (1.67 μM) were added as indicated by the arrows. A, Cu^{2+} added at 0 min; B, Cu^{2+} added at 0 min, histidine at 5 min; C, Cu^{2+} added at 3.2 min, histidine at 6 min; D, Cu^{2+} and histidine added at 0 min; E, control, no additions. These concentrations of additives had no measurable absorbance at 277 nm and the additives produced negligible dilution in the cuvette.

Effect of preincubation with substrate on inhibition

We carried out experiments designed to reveal how much urocanate must be present to produce a Cu^{2+} inhibition which could not be reversed subsequently by histidine as is shown in Fig. 3. The preincubation mixtures contained enzyme, buffer, Cu^{2+} and increasing concentrations of urocanate. After 5 min, histidine was added and the assay was begun with the addition of substrate level (adjusted) of urocanate (Fig. 4). Concentrations of 3.3 μM urocanate or less were not adequate to cause the Cu inhibition which could not be reversed completely by histidine.

About 6.6 μM substrate in the presence of Cu^{2+} and enzyme created the necessary conditions for an inhibition that could not be completely reversed by histidine. Preincubation with low levels of substrate and Cu^{2+} did not reduce the concentration of Cu^{2+} required for 50% inhibition (data not given).

Inhibition of native enzyme

Although the photoactivated enzyme was used primarily in this investigation, several representative experiments with native enzyme were performed to compare to the results just presented. Purified active enzyme was prepared which was not activated by irradiation. Inhibition by Cu^{2+} occurred at the same level of inhibitor; prevention by histidine and EDTA was similar: the results given in Fig. 3 and Table V on order of addition were not different for native enzyme; and the time of preincubation of native enzyme with Cu^{2+} also showed no effect as in Table II. Inhibition by Fe^{2+} was similar in both the native and photoactivated enzymes.

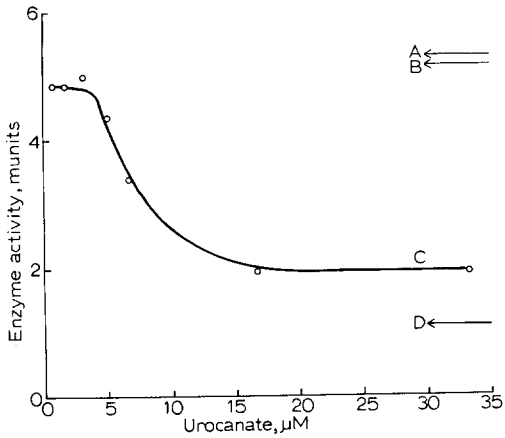

Fig. 4. The effect of urocanate concentration during preincubation with Cu^{2+} on the inhibition of photoactivated urocanase. Assays were performed as described in the text. A, enzyme activity in presence of 1.67 μM Cu^{2+} and 33 μM histidine (added before substrate); B, enzyme activity, no additions; C, enzyme activity after 5 min preincubation with increasing concentrations of substrate in the presence of 1.67 μM Cu^{2+} after which 33 μM histidine, and then substrate level of urocanate were added; D, enzyme activity inhibited by 1.67 μM Cu^{2+}.

DISCUSSION

Low concentrations of Cu^{2+} inhibit urocanase from *P. putida*. Histidine and other metal-binding agents prevent the inhibition. The inhibition is not progressive with time but is manifested immediately when Cu^{2+} is added. These data suggest that metal binding rather than oxidation of sulfhydryl groups accounts for the inhibition. When urocanate is added before the metal-binding agent, the inhibition is not fully reversible. However, metal binding usually should not be influenced by order of addition of reagents. Thus, the nature of the inhibition cannot be described at this time since urocanate seems to be involved in the inhibition. We do not suspect that the product is involved because the inhibition is not related to the time elapsed for product formation (Fig. 3). In conclusion, purified bacterial urocanase has a high and fairly selective sensitivity to inhibition by Cu^{2+}.

ACKNOWLEDGEMENTS

This research was supported by the Veterans Administration, Department of Medicine and Surgery, Research Service, Program 584/01/3795.01/171-06 through the Research and Education Committee at this medical center. We are grateful for the assistance of M. Jean Paige, librarian, in this study and in our research program for several years.

REFERENCES

1 Hug, D. H., Hunter, J. K. and Roth, D. E. (1971) *Photochem. Photobiol.* 13, 171–177
2 Hug, D. H. and Roth, D. (1971) *Biochemistry* 10, 1397–1402
3 Hug, D. H. and Hunter, J. K. (1970) *J. Bacteriol.* 102, 874–876

4 Boothe, R. L. and Folk, J. E. (1969) *J. Biol. Chem.* 244, 399–405
5 Neumann, P. Z. and Sass-Kortsak, A. (1967) *J. Clin. Invest.* 46, 646–658
6 Vallee, B. L. and Wacker, W. C. (1970) in *The Proteins* (Neurath, H., ed.), Vol. V, pp. 129–146 Academic Press, New York
7 Cornell, N. W. and Crivaro, K. E. (1972) *Anal. Biochem.* 47, 203–208
8 Hug, D. H. and Roth, D. (1972) *Photochem. Photobiol.* 16, 203–207