Biochimica et Biophysica Acta, 619 (1980) 267—273

BBA 57613

INHIBITION OF THE MICROSOMAL STEAROYL COENZYME A DESATURATION BY DIVALENT COPPER AND ITS CHELATES

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(Received January 15th, 1980)

Key words: Stearoyl-CoA desaturase; Copper-amino acid chelate; Superoxide scanvenger; (Rabbit, Chicken liver)

Summary

Divalent copper and copper complexes of tyrosine, histidine and lysine inhibited at low concentrations the stearoyl-CoA desaturation reaction in both chicken liver microsomes and in a purified system consisting of chicken liver Δ^9 terminal desaturase, cytochrome b_5, ascorbate and liposome. Although the copper chelates lowered the steady-state level of ferrocytochrome b_5 by 20%, and partially inhibited the NADH-ferricyanide reductase activity, the availability of the ferrocytochrome b_5 during the time course of desaturation was not affected, indicating that the site of inhibition of desaturation was at the terminal step, i.e., on the Δ^9 terminal desaturase. The presence of chelates during catalysis was essential for the observed inhibition. Based on the observation that O_2 is involved in the desaturation and that there is an initial electron reduction of desaturase iron, it is plausible that the copper chelates are inhibiting by acting as superoxide scavengers.

Introduction

The conversion of stearoyl-CoA to oleoyl-CoA in animal liver is catalyzed by a set of microsomal electron transport proteins comprising sequentially of NADH-cytochrome b_5 reductase (flavoprotein), cytochrome b_5 (hemoprotein) and Δ^9 terminal desaturase (non-heme iron containing protein) [1—5]. The overall reaction: Stearoyl-CoA + NADH + H^+ + O_2 → oleoyl-CoA + NAD^+ + 2 H_2O utilizes a molecule of O_2 as an acceptor for two pairs of electrons, one pair derived from the substrate stearoyl-CoA and the other pair from the reduced pyridine nucleotide which is a required coreductant in the reaction. NADH and the reductase can be replaced by ascorbate, although this results in

reduced efficiency of desaturation [2,5]. The desaturase enzyme binds the fatty acyl-CoA and is also believed to be the site of oxygen activation [3,5]. Considerable information is available on the physicochemical properties [3—7] and regulation [5,8—11] of the Δ^9 terminal desaturase, whereas, the information on the mechanism of desaturation is meagre. The only relevent observation on the nature of active species of oxygen in microsomal stearoyl-CoA desaturation is the finding that catalase stimulates this reaction in microsomes [2,13].

In an attempt to understand the nature of active species of oxygen in microsomal stearoyl-CoA desaturation, we have presently examined the effect of divalent copper and its amino acid chelates on the desaturation reaction. Copper complexes have recently been reported by Green et al. [14] to inhibit the formation of free radicals in rat liver microsomes. The results of our present studies indicate that Cu^{2+} and its chelates at low concentration inhibit the overall stearoyl-CoA desaturation in chicken liver microsomes as well as in a reonstituted system composed of cytochrome b_5, ascorbate, purified Δ^9 terminal desaturase and lipid; the site of inhibition being at the Δ^9 terminal desaturase.

Materials and Methods

$Cu(Tyr)_2$, $Cu(Lys)_2$ and $Cu(His)_2$ were prepared at 40°C as described in literature [15] using CuO and the ligands. NADH-cytochrome b_5 reductase was purfied from rabbit liver microsomes [16]. Rabbit liver cytochrome b_5 was a gift from Dr. B.S.S. Masters. Chicken liver Δ^9 terminal desaturase was prepared by Dr. M. Renuka Prasad [5]. The details of other experimental methods are provided in Tables I—III and Figs. 1 and 2. Egg lecithin and dimyristoyl phosphatidylcholine were obtained from P-L Biochemicals. The source of other materials were the same as outlined earlier [5].

Results and Discussion

As can be seen in Fig. 1, divalent copper and its amino acid chelates inhibit the formation of oleyol-CoA in microsomes, although to a different extent. Divalent copper, $Cu(Tyr)_2$ and $Cu(Lys)_2$ are more effective inhibitors than $Cu(His)_2$. The concentration of divalent copper, $Cu(Tyr)_2$ and $Cu(Lys)_2$ required for 50% inhibition of microsomal stearoyl-CoA desaturase activity ranged from 30 to 50 μM. Further, the inhibitory effect of divalent copper and its chelates was more pronounced when ascorbate is used instead of NADH as the source of reducing equivalents (Table I). In addition, divalent copper and its chelates are more efficient inhibitors of oleoyl-CoA formation in a system reconstituted with ascorbate, cytochrome b_5, Δ^9 terminal desaturase and liposomes (Table I). For example, the reconstituted system requires 2 μM $Cu(Tyr)_2$ (cf. Table I) for about 50% inhibition of enzyme activity whereas the microsomal assay using NADH requires 30 μM $Cu(Tyr)_2$ (cf. Fig. 1). As in the microsomal system, $Cu(Tyr)_2$ and $Cu(Lys)_2$ are more effective than $Cu(His)_2$ in inhibition the reconstituted system. Omission of cytochrome b_5 in the reconstituted system does not support desaturation indicating that ascorbate is

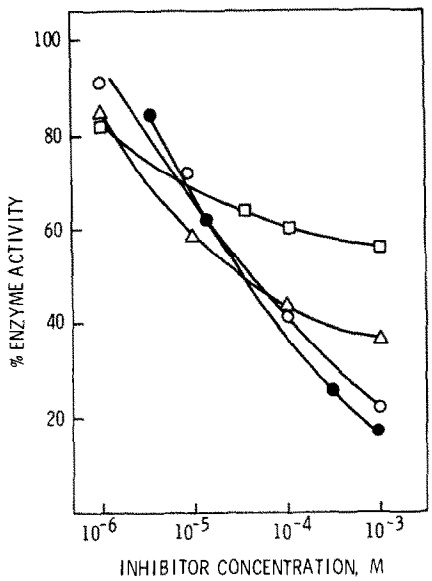

Fig. 1. Inhibition of microsomal stearoyl-CoA desaturase activity by divalent copper and its amino acid chelates. The NADH-supported stearoyl-CoA desaturase activity in chicken liver microsomes was assayed in the absence or presence of various inhibitors using 10 nmol of [1-^{14}C]stearoyl-CoA, 100 nmol of NADH, 40 μmol of potassium phosphate (pH 7.2), 50 μg of microsomal protein and water in a final volume of 0.5 ml [5]. The reaction mixture was incubated in a shaking waterbath at 37°C for 20 min, and the reaction was terminated by the addition of 0.1 ml of 20% methanolic KOH. Fatty acids were extracted and methylated, and the monounsaturated fatty acid methyl esters were separated by thin-layer chromatography on $AgNO_3$-impregnated silica gel H plates. Under the conditions described, the amount of product formed was proportional to the amount of microsomal protein added. The 100% activity corresponds to 2.19 nmol/min per mg. $CuSO_4$ (●); $Cu(Tyr)_2$ (○); $Cu(Lys)_2$ (△); and $Cu(His)_2$ (□).

not able to interact directly with the desaturase enzyme.

When the copper chelates are tested for their effect on NADH-ferricyanide reductase activity in microsomes (Table II) as well as on isolated enzyme (data not shown), the enzyme activity is partially inhibited. A similar effect is also observed with the microsomal NADH-cytochrome *c* reductase activity (data not shown). A recent study indicates that $Cu(Tyr)_2$ also inhibits microsomal NADPH-cytochrome *P*-450 reductase [17,18]. The partial inhibition of the reductase by copper chelates may not be the cause for the inhibition of the microsomal stearoyl-CoA desaturation, since the turnover number of the NADH-cytochrome b_5 reductase is 1000 times higher than the terminal desaturase [13,19]. Also, it has been reported that liver microsomes, in which 99% of NADH-cytochrome b_5 reductase is inhibited by a mercurial, can still show more than 80% of the original desaturase activity [2]. Further, ascorbate supported desaturation which does not involve the reductase enzyme is also inhibited by the copper-chelates (Table I).

The steady-state level of reduced cytochrome b_5 is lowered by copper chelates at concentrations greater than 10 μM. The maximal effect on the reduction of cytochrome b_5 is observed with $Cu(Tyr)_2$ which causes 20% decrease in the steady state level of reduced cytochrome b_5 at 100 μM (Fig. 2A and B). These chelates (100 μM) also enhance the rate of cytochrome b_5 oxidation 2—4-fold when limited amounts of NADH are used (Fig. 2A). This is in agreement with the previous finding that $Cu(Tyr)_2$ stimulates the oxidation of

TABLE I

INHIBITION OF ASCORBATE SUPPORTED STEAROYL-COA DESATURATION BY $CuSO_4$ AND Cu(II)-AMINO ACID CHELATES

Microsome: Ascorbate-supported desaturase activity was assayed as described in legend to Fig. 1, except that 10 μmol of ascorbate was used instead of 100 nmol of NADH. The 100% activity corresponds to 0.24 nmol/min per mg. Reconstituted system: Purified chicken liver stearoyl-CoA desaturase (0.3 μM) was preincubated with rabbit liver cytochrome b_5 (2 μM), ascorbate (20 mM) and 1 mg/ml dimyristoyl phosphatidylcholine or egg lecithin in 0.1 M potassium phosphate buffer, pH 7.2 for 10 min at 37°C in the presence of 0.05% Triton X-100. Aliquots (0.4 ml) were incubated with 10 nmol of [1-^{14}C]stearoyl-CoA in a final volume of 0.5 ml. After incubation for 30 min, the incubation mixture was processed for desaturase activity as described in legend to Fig. 1. The product of desaturation assay in the purified system was confirmed to be oleoyl-CoA by gas chromatographic analysis after conversion into methyl ester [5].

Addition	Concentration (μM)	Ascorbated-supported stearoyl-CoA desaturase Activity in	
		Microsome	Reconstituted system
None		100	100 *,**
$Cu(Tyr)_2$	100	14	0 *
	50	20	—
	10	—	16 *
	2	—	44 **
$Cu(Lys)_2$	100	—	0 *
	50	36	—
	10	—	14 *
	2	—	73 **
$Cu(His)_2$	100	18	0 *
	50	60	—
	10	—	16 **
	2	—	84 **
$CuSO_4$	100	10	—
	10	—	9 **
$CdCl_2$	100	100	—

* Dimyristoyl phosphatidylcholine, 100% activity corresponds to 7.4 nmol/min per mg.
** Egg lecithin, 100% activity corresponds to 18 nmol/min per mg.

TABLE II

EFFECT OF COPPER CHELATES AND EDTA ON MICROSOMAL STEAROYL-CoA DESATURATION AND NADH-FERRICYANIDE REDUCTASE ACTIVITY

The details of the desaturase assay are the same as that described in Fig. 1, except that EDTA (1 mM) is included when indicated prior to the addition of 250 μM $CuSO_4$ or $Cu(Lys)_2$.

Addition	Stearoyl-CoA desaturase activity (NADH-supported) (%)	NADH-ferricyanide reductase activity (%)
None	100 *	100 **
EDTA	100	100
$Cu(Lys)_2$	24	69
$Cu(Lys)_2$ + EDTA	21	105
$CuSO_4$	17	15
$CuSO_4$ + EDTA	37	75

* 100% activity corresponds to 1.9 nmol/min per mg.
** 100% activity corresponds to 3.2 μmol/min per mg.

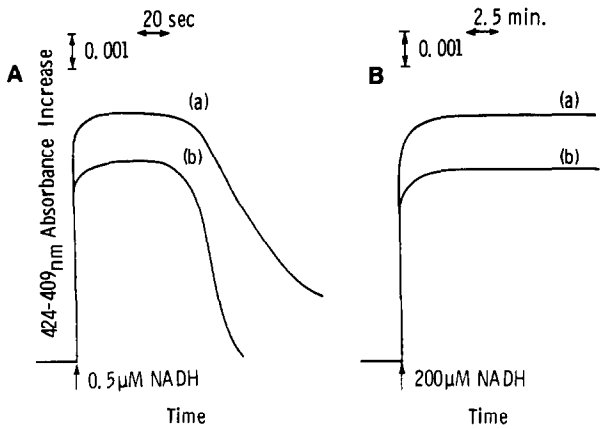

Fig. 2. Steady-state kinetics of microsomal cytochrome b_5. Chicken liver microsomes from fasted-refed animals are diluted to 720 μg/ml into buffer containing 0.1 M Tris-HCl (pH 7.4) and 48 μg/ml Na_2S. (A) Extent of microsomal cytochrome b_5 reduction in aerated buffer on addition of 0.5 μM NADH in the absence (a) and presence (b) of 100 μM $Cu(Tyr)_2$ in a total volume of 1.1 ml. (B) Extent of microsomal cytochrome b_5 reduction in aerated buffer upon addition of 200 μM NADH in a total volume of 1 ml in the presence (b) and absence (a) of 100 μM $Cu(Tyr)_2$. Control experiments under these conditions show that addition of NADH to 100 μM $Cu(Tyr)_2$ alone does not cause any spectral changes. Other experimental details are the same as described before [5].

mircosomal cytochrome b_5 [18,20]. Although, divalent copper and its amino acid chelates decrease the extent of reduction of cytochrome b_5 by 20%, the steady-state levels are maintained (for at least 25 min) in the presence of 200 μM NADH and 20 μM stearoyl-CoA (Fig. 2B). A similar observation is also made for the reduction of cytochrome b_5 by ascorbate in the system reconstituted with purified components (not shown). Thus, during the entire time course of the desaturase assay the availability of reduced cytochrome b_5 for transfer of reducing equivalents to the terminal desaturase is not affected by these inhibitors. These results indicate that the inhibition of stearoyl-CoA desaturation by copper chelates involves the terminal desaturase. The possibility that copper chelates inhibit the desaturase enzyme by reacting with the essential sulfhydryl group(s) of desaturase [5,21] or through their oxidase like activity [22] are excluded based on the observation that EDTA reverses the inhibition of NADH-cytochrome b_5 reductase by copper and its chelates, but does not relieve the inhibition of stearoyl-CoA desaturation (Table II). Further, preincubation of microsomes with copper chelates or $CuSO_4$ (in the absence of ascorbate) does not enhance the inhibitory effect (data not shown). The copper inhibition observed here is not due to heavy metal poisoning of the enzyme since $CdCl_2$ under similar conditions has no inhibitory effect (Table I).

As can be seen in Table III preincubation of the microsomes with copper chelates in the presence or absence of ascorbate and subsequent removal of chelates does not inhibit desaturation. In this respect $CuSO_4$ differs from copper chelates. Pretreatment of the microsomes with $CuSO_4$ in the absence of ascorbate and subsequent removal does not cause inhibition, while pretreatment in the presence of ascorbate causes a significant inhibition. Thus, at least in the case of copper amino acid chelates, it is essential that they be present to obtain

TABLE III

EFFECT OF PRETRATMENT WITH $CuSO_4$ OR $Cu(LYS)_2$ (IN THE PRESENCE OR ABSENCE OF ASCORBATE) AND REMOVAL ON ASCORBATE-SUPPORTED DESATURATION ACTIVITY OF MICROSOME

Microsomes (800 μg/ml) are pretreated with 70 μm $Cu(Lys)_2$ or $CuSO_4$ in the presence or absence of 10 mM ascorbate for 10 min at 37°C. At the end of 10 min, it is diluted 5-fold with the assay buffer and centrifuged at 160 000 *g* for 1 h. The pellet is washed with 5 ml buffer and recentrifuged under the same conditions. The washed pellet is resuspended in 250 μl buffer and aliquots are assayed for desaturase activity in the absence or presence of 70 μM of the inhibitors. Activity of 100% corresponds to 0.9 nmol/min per mg.

Pretreatment and addition	Stearoyl-CoA desaturase activity (%)
Pretreated control + ascorbate	100
+ $CuSo_4$	14
+ $Cu(Lys)_2$	18
Pretreated with $CuSO_4$	89
+ $CuSO_4$	14
Pretreated with $CuSO_4$ + ascorbate	37
+ $CuSO_4$	8
Pretreated with $Cu(Lys)_2$	94
+ $Cu(Lys)_2$	7
Pretreated with $Cu(Lys)_2$ + ascorbate	80
+ $Cu(Lys)_2$	6

inhibition. In addition to the thiol oxidation and oxidase like activities, copper and certain copper chelates are reported to have catalatic [23], peroxidative [23] and dismutative [24,25] activities. Since catalase is known to stimulate desaturation [12,13], it is probable that copper chelates are not affecting desaturation through their catalatic or peroxidative activities, both of which would lead to breakdown of H_2O_2. The possibility that they are acting as superoxide scavengers appears plausible in view of the following observations: (a) the initial single electron reduction of desaturase iron and involvement of O_2 [3]; (b) microsomes produce superoxide in the presence of NADH and O_2 [26]; (c) in addition to stearoyl-CoA and certain other long chain fatty acid acyl-CoAs [4,5], *p*-cresol and several other phenols also interact with the desaturase system causing a stimulation of oxidation of reduced cytochrome b_5 [27]; *p*-cresol is known to be hydroxylated in the presence of superoxide generating system [28]. Since *p*-cresol does not bear any structural similarity to stearoyl-CoA, it may be interacting with an active species (possibly superoxide) generated by the desaturase system; and finally (d) stearoyl-CoA desaturation is inhibited by free radical scavengers 5,5-dimethyl-1-pyrroline-*N*-oxide (superoxide and hydroxyl radical trap) and adrenaline (superoxide scavenger), (unpublished results). However, confirmation of this possibility must await further experimental proof. In any case, understaing the mechanism by which copper chelates act may throw light on the mechanism of stearoyl-CoA desaturation.

Acknowledgements

We thank Dr; Salih J. Wakil for his interest and for providing laboratory facilities. We also thank Drs. R.W. Estabrook and J. Werringloer for their

useful suggestions. This investigation was supported by United States Public Health Service grant HD07516. V.C.J. is a recipient of United States Public Health Service Research Carreer Development Award AM00397.

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