THE EFFECT OF A TYROSINE–DIVALENT COPPER COMPLEX ON HEPATIC MICROSOMAL DEMETHYLATION

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1. Introduction

The mechanism of oxygen activation by cytochrome *P*-450 of hepatic microsomes during the mixed-function oxidation reactions has not been clarified.

Since superoxide radicals, O_2^-, were indicated as possible intermediates in the reaction [1,2], erythrocuprein was tested as a potential inhibitor of the mixed-function oxidase.

Erythrocuprein proved not to be effective on benzphetamine hydroxylation of intact rat liver microsomes, but inhibited hydroxylation by purified cytochrome *P*-450 and its reductase [3]. In this case the reductase could be substituted by a superoxide radical generating system [3].

The observations reported above left open two possible interpretations: (1) superoxide radicals are not involved in the mixed function oxidase reaction in an intact system; (2) superoxide radicals are involved in the mixed function oxidase reaction of intact microsomes, but they are not available to the action of external erythrocuprein.

A choice between the two alternatives left open by Strobel and Coon's experiments [3] may be afforded by the study of the effect on microsomal mixed-function oxidases of low molecular weight copper complexes provided with superoxide dismutase activity [4].

It was found that Tyr_2–Cu^{2+} complex inhibits, at low concentrations, *p*-nitroanisol and aminopyrine demethylation. Free tyrosine, Cu^{2+} and Lys–Cu^{2+} complex were not effective at the same concentrations.

No evidence was found that Tyr_2–Cu^{2+} complex inhibited cytochrome b_5 reduction, NADPH-cytochrome *c* reductase or substrate binding. Tyr_2–Cu^{2+} complex did not behave as an hydroxylatable substrate or as an electron acceptor from cytochromes b_5 or *P*-450.

The data reported in this study suggest that the inhibitory effect of Tyr_2–Cu^{2+} complex is related to its superoxide dismutase activity, exerted in a scarcely accessible, low polarity environment. They imply that O_2^- radicals are a species of direct importance in the mixed-function oxidase reactions.

2. Materials and methods

Male Wistar rats, 150–200 g were induced with daily intraperitoneal injections of 80 mg phenobarbital per kg body weight for three days, followed by 24 h fasting.

Microsomes were prepared according to a published method [5].

p-Nitroanisol *O*-demethylation [6] was measured in a dual-wavelength spectrophotometer (constructed in the workshop of the Johnson Research Foundation, University of Pennsylvania, Philadelphia, USA) using 417 and 480 nm as measuring and reference wavelength to follow the formation of *p*-nitrophenol, and the extinction coefficient ϵ mM = 13.2 at 417–480 nm The incubation system consisted of 150 mM KCl, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 7 mM isocitrate, 1 mM NADP and enough isocitrate dehydrogenase to reduce 60 nmol of NADP per min/ml. For aminopyrine *N*-demethylation, the same medium was used except that glucose-6-P and glucose-

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6-P dehydrogenase were used as a regenerating system for NADPH. Semicarbazide (1 mM) was also present. Formaldehyde determination was carried out by the method of Nash [7].

Cytochrome *c* reductase was measured in a Hitachi-Perkin-Elmer spectrophotometer (Model 124) in 150 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 0.1 mM ferricytochrome *c*, 0.3 mg microsomal protein/ml, and the reaction was started with 1 mM NADPH. Ferrocytochrome *c* formation was measured at 550 nm in a Hitachi-Perkin-Elmer spectrophotometer (Model 124). Cytochrome b_5 reduction was measured in the regenerative flow apparatus (constructed in the Johnson Research Foundation, University of Pennsylvania, Philadelphia, USA), operated in the continuous flow mode [8]. Microsomes were incubated in the buffer employed for cytochrome *c* reductase, except that cytochrome *c* was omitted and the reaction initiated by different amounts of NADPH. Mitochondrial fragments utilized for reaching and maintaining anaerobic conditions were prepared by the method of Beyer [9]. Protein concentration was measured by the biuret method [10].

All reagents employed were commercial samples of the highest available purity. The complexes of copper with different amino acids, prepared in a way similar to the method given in [11,12], were a generous gift of Dr U. Weser, University of Tübingen.

3. Results

3.1. *Inhibition of Tyr_2–Cu^{2+} of p-nitroanisol and aminopyrine demethylation*

The effect of Tyr_2–Cu^{2+} on the demethylation of *p*-nitroanisol was measured at low and high substrate concentration. The formation of the product, *p*-nitrophenol, was followed spectrophotometrically. The concentration of Tyr_2–Cu^{2+} producing 50% inhibition was approximately 10 and 17 μM at low and high substrate concentration, respectively (fig.1).

Aminopyrine demethylation was found to be 85% inhibited by 10 μM Tyr_2–Cu^{2+} at a concentration of 1 mM substrate. The formaldehyde formed in the reaction was measured after 10 min incubation of 1 mg/ml microsomal protein in the presence and absence of the copper complex.

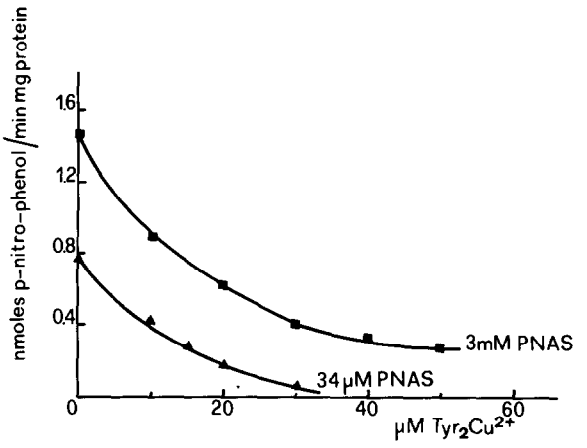

Fig.1. Effect of Tyr_2–Cu^{2+} on the *O*-demethylation of *p*-nitroanisol to *p*-nitrophenol. Microsomes from phenobarbital induced rats were diluted to 1 mg/ml into buffer containing 150 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 7 mM isocitrate, 1 mM NADP and various amounts of Tyr_2–Cu^{2+} in a total of 2 ml. The reaction was started by adding an amount of isocitrate dehydrogenase sufficient to reduce 60 nmol of NADP per minute and ml. The formation of *p*-nitrophenol was followed in a dual-wavelength spectrophotometers at 417–480 nm at room temperature. PNAS = *p*-nitroanisol.

3.2. *Changes in the kinetics of microsomal pigments produced by Tyr_2–Cu^{2+}*

The steady state of microsomal pigments was measured in the dual-wavelength spectrophotometer at 557–540 nm, in an air saturated buffer. After addition of limiting amounts of NADPH (20 μM), a reduction of microsomal pigments was observed, followed by reoxidation after NADPH exhaustion, (fig.2A, curve a). As indicated by Estabrook et al. [13] the presence of a substrate produced a lower steady state and a faster reoxidation kinetics of microsomal pigments (curve b).

Tyr_2–Cu^{2+} (50 μM) (curve c) was more efficient than the substrate in lowering the state of microsomal pigment reduction as well as in stimulating their rate of oxidation. On the assumption that cytochrome b_5 is the main absorbing species at the pair of wavelength employed, and that its total concentration is 0.55 nmol/mg protein a turnover number can be calculated using the equation derived by Chance [14] and applied to microsomal pigments by

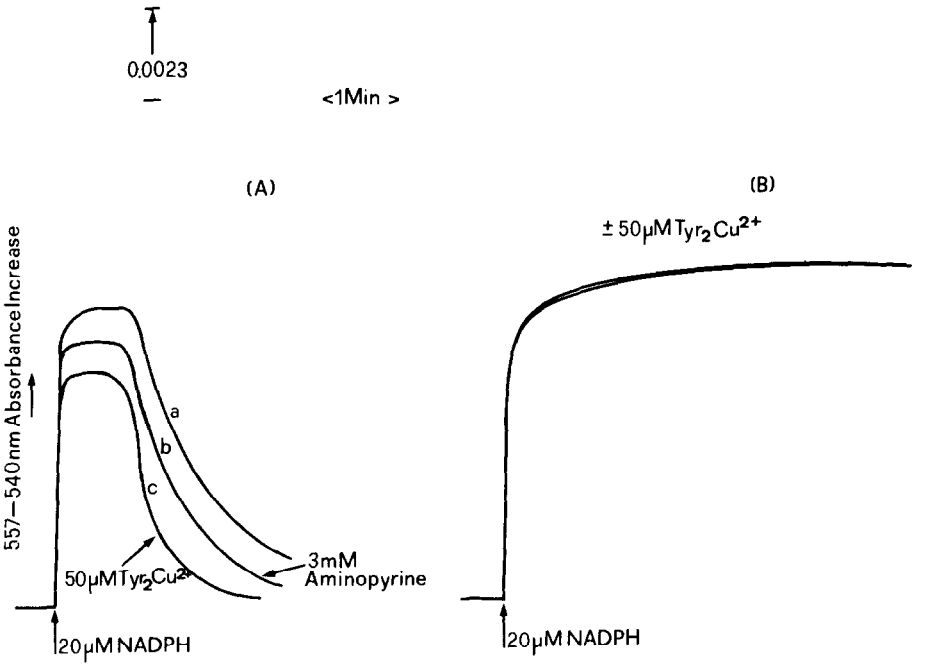

Fig.2. Steady state kinetics of microsomal pigments. Microsomes from phenobarbital induced rats were diluted to 1 mg/ml into buffer containing 150 mM KCl, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 10 mM nicotinamide. (A) Extent of microsomal pigment reduction in air-saturated buffer upon addition of 20 μM NADPH in a total of 2 ml: Curve a, no substrate present; curve b, in the presence of 3 mM aminopyrine; curve c, in the presence of 50 μM Tyr_2-Cu^{2+}. (B) Extent of microsomal pigment reduction in anaerobic buffer upon addition of 20 μM NADPH in the presence and absence of Tyr_2-Cu^{2+} in a total of 3.5 ml. Anaerobiosis was obtained by the presence of 0.9 mg/ml of mitochondrial particles and 3 mM succinate. Control experiments showed that addition of NADPH to mitochondrial particles in the absence of microsomes did not result in spectral changes under these conditions.

Modirzadeh and Kamin [15]. The presence of aminopyrine or Tyr_2-Cu^{2+} appear to increase the turnover number of cytochrome b_5 from 0.039 sec^{-1} to 0.055 sec^{-1} and 0.079 sec^{-1} respectively.

In fig.2B, when strict anaerobiosis was reached and maintained by adding mitochondrial fragments and succinate to the incubation medium, the reduction of microsomal pigments was the same in the presence and absence of Tyr_2-Cu^{2+}. This observation indicates that the stimulation by Tyr_2-Cu^{2+} of the oxidation of microsomal pigments requires the presence of oxygen, and thus is not a direct oxidation of microsomal pigments by Tyr_2-Cu^{2+}.

Since the decrease in the steady-state of reduction of microsomal pigments produced by Tyr_2-Cu^{2+} could be the consequence of an inhibition of the reducing pathways, NADPH cytochrome *c* reductase and the rate of cytochrome b_5 reduction were measured in the presence and absence of the copper complex.

As shown in table 1, neither cytochrome b_5 reduction rate, nor NADPH-cytochrome *c* reductase were significantly affected by Tyr_2-Cu^{2+}.

3.3. *The effect of different compounds having superoxide dismutase activity on* p*-nitroanisol demethylation and cytochrome* P*-450 content of hepatic microsomes*

$CuSO_4$ [4], amino acid–Cu^{2+} complexes [4], and EDTA–Fe^{2+} [16] are compounds provided with superoxide dismutase activity. They were tested (table 2) for their ability to inhibit *p*-nitroanisol demethylation. At the same time their efficiency in transforming cytochrome *P*-450 to cytochrome *P*-420 was tested, since such a transformation could be a

Table 1
The effect of Tyr_2–Cu^{2+} on the rate of microsomal cytochromes reduction and on NADPH-cytochrome *c* reductase

NADPH (μM)	NADPH-cyt. *c* reductase (nmoles/min/mg)		Reduction of microsomal cytochromes (sec^{-1})	
	–Tyr_2–Cu^{2+}	+Tyr_2–Cu^{2+}	–Tyr_2–Cu^{2+}	+Tyr_2–Cu^{2+}
10	–	–	331	301
200	–	–	366	310
1000 + regenerating system	150	140	–	–

NADPH-cytochrome *c* reductase activity was measured by adding 1.8 mg of phenobarbital-induced rat liver microsomes into 6 ml buffer containing 150 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 0.1 mM ferricytochrome *c*. The suspension was equally devided into the two cuvettes of a Hitachi-Perkin-Elmer spectrophotometer (Model 124) and the reaction started by the addition of 1 mM NADPH to the sample cuvette. Microsomal pigment reduction was measured at 557–540 nm in a regenerative flow apparatus as described in [8]. Protein concentration was about 1 mg/ml. The concentration of Tyr_2–Cu complex when present was 50 μM.

Table 2
Effect of various compounds with superoxide dismutase activity on microsomal *O*-demethylation and on transformation of cytochrome *P*-450 to cytochrome *P*-420

	nmoles PNP/min/mg protein	Inhibition (%)	*P*-450–CO complex (nmoles/mg protein)	Transformation to *P*-420 (%)
No inhibitor	0.62	–	1.79	–
50 μM $CuSO_4$	0.55	11.3	1.62	9.5
2.5 μM erythrocuprein	0.64	0.0	1.79	0.0
50 μM $FeSO_4$ + 100 μM EDTA	0.49	21.0	1.62	9.5
55 μM Lys–Cu^{2+}	0.64	0.0	n.m.	n.m.
115 μM Lys–Cu^{2+}	0.42	32.3	n.m.	n.m.
220 μM Lys–Cu^{2+}	0.36	42.0	1.70	4.1
50 μM Tyr_2–Cu^{2+}	0.00	100.0	1.73	3.4
Tyrosine (saturated)	0.62	0.0	1.79	0.0

The formation of *p*-nitrophenol from *p*-nitroanisol (34 μM) was determined in the presence of the indicated compounds as described in the legend of fig.1. For the determination of the transformation of cytochrome *P*-450 to cytochrome *P*-420 8 mg of microsomal proteins were diluted into 6 ml of buffer containing 150 mM KCl, 50 mM Tris-KCl pH 7.8 and 10 mM $MgCl_2$ and reduced by the addition of few grains of dithionite. The suspension was divided equally into two cuvettes and the sample cuvette was bubbled for 1 min with CO. The content of cyt. *P*-450 was calculated using a ϵ mM of 91 at 450–490 nm. n.m. = not measured; PNP ≈ *p*-nitrophenol.

trivial explanation for the inhibition of the mixed-function oxidase reduction.

$CuSO_4$, one of the most active catalysts of the dismutation of superoxide radicals, gave only a small inhibition of the demethylation of *p*-nitroanisol, at low substrate concentration, where the inhibition should be more effective (cf. fig.1). The inhibition can be accounted for by a partial transformation of *P*-450 to *P*-420. Erythrocuprein, as already known [2], was not an inhibitor of demethylase activity in intact microsomes, while the complex of Fe^{2+} with EDTA was only slightly inhibitory, if one considers that part of the inhibition of *p*-nitrophenol formation can be explained in terms of *P*-450 transformation. Of the two copper complexes studied, Tyr_2-Cu^{2+} was much more effective than $Lys-Cu^{2+}$. Both complexes gave a very small transformation of *P*-450, not proportional to the inhibition of the demethylation process.

4. Discussion

The use of a number of compounds provided with superoxide dismutase activity, as potential inhibitors of hepatic microsomes demethylations, is amply justified by the recurrent hypotheses involving superoxide radicals as an intermediate in mixed-function oxidase reactions.

The finding that a low molecular weight divalent copper complex with tyrosine inhibits microsomal demethylations can be considered as evidence (although indirect) that O_2^- radicals are involved in the process, provided that none of the electron transfer reactions leading to cytochrome *P*-450 and b_5 reduction are inhibited. It has been shown that neither b_5 nor cytochrome *c*-reductase activities were affected by concentration of Tyr_2-Cu^{2+} fully inhibiting microsomal demethylation. It has also been shown that *P*-450 itself is not modified by the complex.

It was also excluded that Tyr_2-Cu^{2+} could inhibit by competing for the substrate. In fact, no change of the characteristic aminopyrine-*P*-450 spectrum was observed in the presence of 50 μM Tyr_2-Cu^{2+} nor was the copper complex able to bind to cytochrome *P*-450 producing a spectral change.

The possibility that Tyr_2-Cu^{2+} complex acted as an electron acceptor at the level of microsomal pigments was discarded since they were equally reduced (in the absence of oxygen) in the presence and absence of the complex. If Tyr_2-Cu^{2+} acted as an electron sink either at *P*-450 or b_5 level a more oxidized state would be expected in the absence of O_2.

Positive evidence in favour of the concept that Tyr_2-Cu^{2+} acted as superoxide dismutase, comes from the experiments of fig.2 indicating that in the presence of oxygen the steady state of microsomal cytochromes was more oxidized when the complex was present than in its absence. This implies that only in the presence of oxygen, the complex was able to withdraw electrons from the system, as expected in a superoxide dismutase reaction.

In conclusion, the site of action of the Tyr_2-Cu^{2+} complex in inhibiting microsomal demethylations appears to be close or at *P*-450 level. The similarity between the effect of the substrate and that of the complex on the steady state of microsomal cytochromes reduction together with the requirement of oxygen for such an effect point strongly to an inhibitory mechanism of Tyr_2-Cu^{2+} related to its dismutase activity. The finding that neither Cu^{2+}, nor the polar $Lys-Cu^{2+}$ or Fe^{2+}–EDTA complexes or the large erythrocuprein molecule are effective inhibitors of the demethylation reaction, points to the concept that superoxide radicals are produced in an environment not accessible to large and/or polar molecules.

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