REACTIVITY OF SUPEROXIDE DISMUTASE-ACTIVE Cu(II) COMPLEXES ON THE RATE OF ADRENOCHROME FORMATION

Maged YOUNES and Ulrich WESER
Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Str. 1, 74 Tübingen, GFR

Received 25 September 1976

1. Introduction

Since the discovery that cuprein (Cu,Zn-superoxide dismutase) inhibited the oxidation of epinephrine by the xanthine–xanthine oxidase-system [1,2] as well as its autoxidation [3], many workers regarded the superoxide anion radical as the agent responsible for the generation of adrenochrome. The participation of $^{\cdot}O_2^-$ in a reaction was postulated whenever a co-oxidation of epinephrine was observed, which could be inhibited by cuprein. This way the autoxidation of hemoglobin [4,5] was said to be mediated by superoxide which was also believed to be involved in hemichrome precipitation [5]. Goldberg and Stern used the same method to prove the generation of superoxide during the interaction of phenylhydrazine with hemoglobin [6] and during the oxidation of hemoglobin by menadione [7]. Rotilio et al. [8] observed an oxidation of epinephrine during the decay of oxyperoxidase and postulated the participation of free superoxide anions in this reaction. Forman and Fridovich took the adrenochrome formation during the electrolysis of oxygen-saturated buffered aqueous solutions as an evidence that the superoxide anion radical, which is produced electrolytically, could migrate in aqueous solutions to react with small molecules [9].

Using the pulse radiolysis technique Bors et al. [10] could show that the adrenochrome formation occurs via a complicated mechanism and is not a simple reaction between superoxide and epinephrine. It was of interest, thus, to examine whether or not the adrenochrome generation is in fact only due to the action of the superoxide anion radical. If it were so, low molecular weight Cu(II) chelates which proved as good superoxide dismutases as the native Cu,Zn-protein itself [11,12] should inhibit the oxidation of epinephrine in a way similar to cuprein.

However, a contrary action was observed: all copper chelates accelerated the rate of adrenochrome formation. The acceleration rate was a logarithmic function of the copper concentration. Scavengers of both singlet oxygen and hydroxyl radicals had no effect, but H_2O_2 inhibited the oxidation of epinephrine completely. This inhibitory action was overcome by the addition of catalase.

2. Materials and methods

Catalase, xanthine oxidase and xanthine were from Boehringer, Mannheim, glycine, sodium salicylate, $CuSO_4$, CuO, H_2O_2, l-lysine, l-tyrosine and DABCO (diaza-bicyclo-(2,2,2)-octane) from Merck, Darmstadt. Xanthine oxidase was freed from contaminating components by gel filtration (Sephadex G-75). The concentration was calculated using $\epsilon_{280} = 2.04 \times 10^3$ M^{-1} cm^{-1} [13]. Cu,Zn-superoxide dismutase was isolated following the procedure of Weser et al. [14]. The Cu(II) chelates of amino acids [11,12] or salicylate [15] were prepared as described elsewhere [11,12,15]. The formation of adrenochrome was followed at 480 nm using a Unicam SP 1800 spectrophotometer.

2.1. *The autoxidation of epinephrine*

The assay was carried out in a way similar to that given in [2,16]. In a total volume of 700 μl were present: oxygen-saturated glycine buffer, 0.1 M, pH 10.0, epinephrine, 5.7×10^{-4} M and the copper

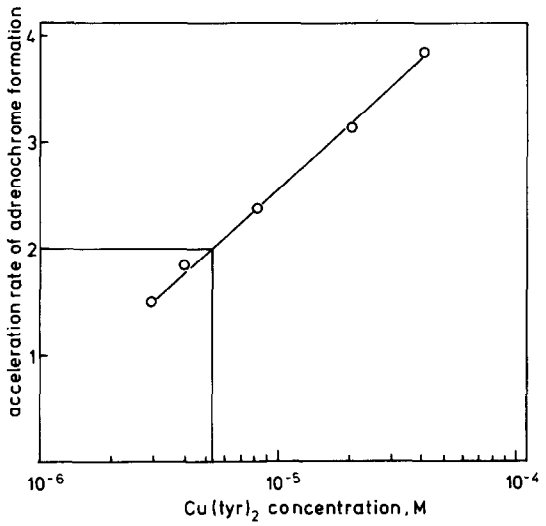


Fig.1. Acceleration rate of the adrenochrome formation by $Cu(tyr)_2$ as a function of the logarithm of the Cu^{2+} concentration. For experimental details see legend to table 1.

complex under investigation. The acceleration rate of the adrenochrome formation by different amounts of each copper complex were determined and plotted against the logarithm of the copper concentration. Linear plots were obtained allowing the determination of the copper concentration needed to double the velocity of the epinephrine autoxidation. Figure 1 shows such a plot for $Cu(tyr)_2$.

2.2. *The oxidation of epinephrine mediated by the xanthine-xanthine oxidase system*

The assay conditions were those described by McCord and Fridovich [1]. The assay mixture was composed of glycine buffer, 50 mM, pH 10.0, xanthine, 1.1×10^{-4} M, epinephrine 4.45×10^{-4} M and xanthine oxidase, 1.1×10^{-8} M. The reaction was started by the addition of xanthine oxidase. The total volume was 900 μl. The concentrations of the different Cu(II) chelates needed to double the rate of adrenochrome formation were determined graphically as described above.

3. Results

3.1. *The effect of different Cu(II) chelates on the rate of adrenochrome formation*

Copper chelates, which are able to catalyze the spontaneous dismutation of the superoxide anion at a rate which exceeds that shown by native Cu,Zn-superoxide dismutase, were, unlike native cuprein, able to suppress neither the autoxidation of epinephrine nor the xanthine–xanthine oxidase-mediated oxidation of it. On the contrary, the rate of adrenochrome formation was accelerated in both cases in the presence of the Cu(II) chelates. The rate of acceleration proved a linear function of the logarithm of the Cu(II) concentration (fig.1). Table 1 lists the concentrations of the different Cu(II) complexes needed to double the velocity of the epinephrine autoxidation reaction. Table 2 shows the same data for the xanthine–xanthine

Table 1
The ability of different superoxide dismutase-active Cu(II)-chelates to accelerate the autoxidation of epinephrine

Copper chelate	Concentration able to double the velocity of adrenochrome formation (μM)
Cu^{2+} aq	11.2
$Cu(salicylate)_2$	11.2
$Cu(lys)_2$	9.6
$Cu(tyr)_2$	5.3

The assay mixture contained glycine buffer, 0.1 M, pH 10.0, epinephrine, 5.7×10^{-4} M and different copper chelates in a total volume of 700 μl. The concentration of the copper chelates able to double the velocity of adrenochrome formation were determined graphically. The reaction was started by adding the epinephrine solution, then the adrenochrome formation was followed spectrophotometrically at 480 nm.

Table 2
Effect of low molecular weight copper chelates on the xanthine–xanthine oxidase-mediated oxidation of epinephrine

Cu(II) chelate	Concentration able to double the velocity of adrenochrome formation (μM)
Cu^{2+} aq	17.4
$Cu(salicylate)_2$	16.0
$Cu(lys)_2$	10.5
$Cu(tyr)_2$	8.6

In a total volume of 900 μl were present: glycine buffer, 50 mM, pH 10.0, xanthine, 1.1×10^{-4} M, epinephrine, 4.45×10^{-4} M and xanthine oxidase 1.1×10^{-8} M. The reaction was started by the addition of xanthine oxidase and the formation of adrenochrome was monitored at 480 nm.

oxidase-mediated oxidation of adrenaline. In both cases only micromolar amounts of $Cu(tyr)_2$, for example, were needed to double the rate of adrenochrome formation.

3.2. *Effect of scavengers of singlet oxygen and hydroxyl radicals*

The contradictory behaviour of cuprein on the one side and the low molecular weight copper chelates on the other side, all known to be good superoxide dismutases, could be thought of in terms of some reactive species being generated during or following the disproportionation of superoxide which is responsible for the oxidation of epinephrine and the generation of which is only suppressed in the case of native Cu,Zn-superoxide dismutase. The reaction of $^{\cdot}O_2^-$ with H_2O_2, for example, might generate hydroxyl radicals ($^{\cdot}OH$) as well as singlet oxygen ($^1\Delta_gO_2$), as proposed by Kellogg and Fridovich [17]:

$$^{\cdot}O_2^- + H_2O_2 \longrightarrow OH^- + {}^{\cdot}OH + O_2^*$$

To examine this possibility, DABCO (diaza-bicyclo-(2,2,2)octane), a well established water-soluble singlet oxygen quencher and tert-butanol, a potent scavenger of $^{\cdot}OH$-radicals, were added to the assay mixture in different concentrations ranging from 1–10 mM in the presence as well as in the absence of the copper complexes. No effect of either reagent could be observed in any case, even in ^2H_2O, showing that these reactive oxygen species are most probably not responsible for the adrenochrome formation.

3.3. *Action of H_2O_2 and catalase*

When catalase (6.2 μM) was added to the assay mixture in the presence and in the absence of the copper chelates the rate of epinephrine autoxidation was not affected, but in either case more epinephrine was oxidized in the presence of catalase. This suggested an inhibitory action of accumulated H_2O_2 on the adrenochrome formation being overcome by the addition of catalase. As a matter of fact H_2O_2 (7.85×10^{-4} M) inhibited the adrenochrome formation completely in the absence of the Cu(II) chelates and in the presence of either cuprein or $Cu(tyr)_2$. When catalase was added, a dramatic and rapid rise in the absorption at 480 nm was observed suggesting that some reactive species able to oxidize epinephrine was generated during the removal of H_2O_2 by catalase. This species can neither be the hydroxyl radical nor singlet oxygen, since *tert*-butanol as well as DABCO, both added up to a final concentration of 10 mM had no influence on the observed phenomena even in ^2H_2O. Table 3 lists some of the observed phenomena.

4. Discussion

The fact that the low molecular weight Cu(II) chelates which possess a superoxide dismutase activity accelerated the adrenochrome formation instead of inhibiting this reaction like native cuprein, suggests that the inhibitory action of Cu,Zn-superoxide dismutase on the epinephrine oxidation might not be due to the quenching of the superoxide anion radical.

Table 3
The effect of peroxide and catalase on the autoxidation of epinephrine

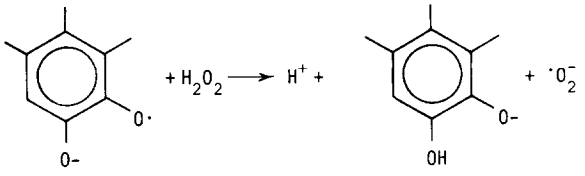
Additions (μM)	Acceleration rate of adrenochrome formation	Quantity of adrenochrome formed (rel.)
None	1.0	1.0
H_2O_2 (78.5)	0.0	0.0
H_2O_2 (78.5) + Catalase (0.0062)	49.2	22.8
$Cu(tyr)_2$ (20.25)	2.2	1.1
$Cu(tyr)_2$ (20.25) + Catalase (0.0062)	2.3	1.4
$Cu(tyr)_2$ (20.25), Catalase (0.0062) + H_2O_2 (78.5)	49.6	23.0

The assay conditions are the same as in the legend to table 1. The acceleration rate and the end concentration of adrenochrome in the absence of all additions were defined as 1.0.

Rapp et al. performed EPR experiments which showed that native cuprein could react with different catechols in air giving rise to altered EPR-spectra reflecting an alteration of the Cu(II) environment [18]. The semiquinone of epinephrine, which is not detectable in aqueous solution, could be detected in the presence of cuprein at 77°K. They could show that cuprein causes the accumulation of much higher concentrations of some catechol radicals than is possible by air alone.

On the other hand, Walaas et al. [19] showed that Cu(II) ions reacted with epinephrine under anaerobic conditions yielding a 1:2 (Cu/epinephrine) complex; the complex formation was favoured at higher pH. In the presence of oxygen an accelerated adrenochrome formation was observed which could be abolished by EDTA but not influenced by cyanide.

In the present study not only free Cu^{2+} ions accelerated the adrenochrome formation, but also stable Cu(II) chelates. The inhibitory action of peroxide on the epinephrine oxidation might be due to a reaction between the adrenaline semiquinone and H_2O_2, e.g.



But the effect of catalase added to the assay mixture containing H_2O_2 is not easy to understand. Some very reactive species might be produced during the catalytic destruction of peroxide by catalase, even if it were bound to the enzyme, e.g. $\cdot O_2^-$. The acceleration of the adrenochrome formation by the Cu(II) chelates can be explained by the ability of Cu^{2+} to withdraw an electron from epinephrine-bound oxygen. Thus, a semiquinone is formed which is immediately oxidized to yield adrenochrome.

In any case, the main conclusion that can be drawn from all these observations is that the adrenochrome formation is not a simple reaction between $\cdot O_2^-$ and epinephrine. Therefore, conclusions that superoxide is involved in a reaction whenever a co-oxidation of epinephrine is observed which can be inhibited by superoxide dismutase, should not be drawn until the mechanism of this reaction is proven.

Acknowledgements

This study was aided by DFG grants on copper proteins to U.W. M.Y. is a recipient of a German Academic Exchange Service (DAAD) fellowship.

References

[1] McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[2] Valerino, D. M. and McCormack, J. J. (1969) Federation Proc. 28, 545.
[3] Misra, H. P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175.
[4] Misra, H. P. and Fridovich, I. (1972) J. Biol. Chem. 247, 6960–6962.
[5] Brunori, M., Falcioni, G., Fioretti, E., Giardina, B. and Rotilio, G. (1975) Eur. J. Biochem. 53, 99–104.
[6] Goldberg, B. and Stern, A. (1975) J. Biol. Chem. 250, 2401–2403.
[7] Goldberg, B. and Stern, A. (1976) Biochim. Biophys. Acta 437, 628–632.
[8] Rotilio, G., Falcioni, G., Fioretti, E. and Brunori, M. (1975) Biochem. J. 145, 405–407.
[9] Forman, H. J. and Fridovich, I. (1972) Science 175, 339.
[10] Bors, W., Saran, M., Michel, C., Lengfelder, E., Fuchs, C. and Spöttl, R. (1975) Int. J. Radiat. Biol. 28, 353–371.
[11] Brigelius, R., Spöttl, R., Bors, W., Lengfelder, E., Saran, M. and Weser, U. (1974) FEBS Lett. 47, 72–75.
[12] Brigelius, R., Hartmann, H. J., Bors, W., Lengfelder, E., Saran, M. and Weser, U. (1975) Z. Physiol. Chem. 356, 739–745.
[13] Massey, V., Brumby, P. E., Komai, H. and Palmer, G. (1969) J. Biol. Chem. 244, 1682–1691.
[14] Weser, U., Bunnenberg, E., Cammack, R., Djerassi, C., Flohé, L., Thomas, G. and Voelter, W. (1971) Biochim. Biophys. Acta 243, 203–213.
[15] de Alvare, L. R., Goda, K. and Kimura, T. (1976) Biochem. Biophys. Res. Comm. 69, 687–694.
[16] Paschen, W. (1972) Biochemical Master Thesis, Tübingen.
[17] Kellogg, E. W. and Fridovich, I. (1975) J. Biol. Chem. 250, 8812–8817.
[18] Rapp, U., Adams, W. C. and Miller, R. W. (1973) Can. J. Biochem. 51, 158–171.
[19] Walaas, E., Walaas, O. and Haavaldsen, S. (1963) Arch. Biochem. Biophys. 100, 97–109.