Biochemical Oxygen Activation as the Basis for the Physiological Action of Tetrachlorodecaoxide (TCDO)

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Oxidation of methionine, 4-(methylthio)-2-oxobutyric acid (KMB), or 1-aminocyclopropane carbonic acid (ACC) are indicator reactions for activated oxygen species such as singlet oxygen ($^1O_2$), OH$^\cdot$-radical like oxidants, superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) or activated hemo-iron complexes like peroxidase- or catalase-"compound I". Methionine is oxidized by OH$^\cdot$ as well as by $^1O_2$ forming ethylene, but not by tetrachloro-decaoxygen complex (TCDO) in the absence or presence of catalytic hemoproteins such as peroxidase, hemoglobin or myoglobin. Both KMB and ACC are oxidized by TCDO under the catalysis of the above hemo-proteins where neither catalase nor superoxide dismutase are inhibitors. TCDO hemo-protein complex is an oxidant with similar properties as peroxidase-compound I and can clearly be differentiated from $O_2^{\cdot -}$, H_2O_2, OH$^\cdot$ and $^1O_2$.

Introduction

Oxoferin^R contains a complex, inorganic chlorine-oxygen compound (TCDO: Tetrachloro-decaoxygen complex) with the empirical formula $(Cl_4O_{10}.H_2O)^-$. It possesses antibacterial activity [1] and is commercially available since early 1984, indicated for use in secondary wound healing [2]. We reported on some biochemical oxidative properties of the compound, activated by certain heme-iron proteins such as hemoglobin, myoglobin or peroxidase [3]. In order to gain a better insight into the mechanism of the physiological properties of this substance, studies were undertaken so that

1) the biochemical reactivity of the oxygen complex could be determined and
2) a proposal for the possible molecular mechanism of action *in vivo* could be made.

Abbreviations: OF, Oxoferin^R; SOD, superoxide dismutase; POD, peroxidase; Met, methionine; KMB 4-(methylthio)-2-oxobutyric acid; ACC, 1-aminocyclopropane carboxylic acid; AQ anthraquinone-2-sulphonate; XOD, xanthine oxidase; TCDO, Tetrachloro-decaoxygen complex.

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A) *Assay systems*

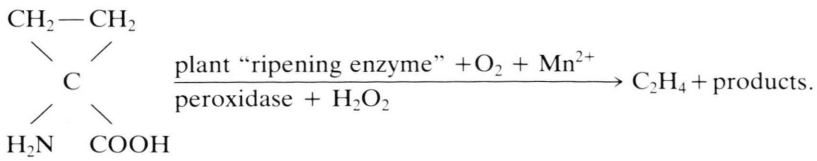
With the aid of gas chromatographic detection of the breakdown of certain indicator molecules forming ethylene, it is possible to determine the oxidizing ability of a range of oxygen species. The aim of these studies was to differentiate between potentially toxic and active oxygen species which occur as a result of normal metabolism. Through the use of the indicator molecules ACC, KMB and MET it is possible to distinguish between extremely reactive and hence potentially toxic species such as $^1O_2$, OH$^\cdot$ and $HO^\cdot_2$ and metabolic intermediates such as H_2O_2, compound I of catalase and peroxidase, and $O_2^{\cdot -}$. The respective oxidation rates of these substances are characteristic for each of the oxidants in addition to the effects of a range of inhibitors on these reactions.

1) Oxidation of 4-(methylthio)-2-oxobutyric acid (KMB)

$$CH_3S-CH_2CH_2-COCOOH \xrightarrow[\text{peroxidase} + H_2O_2]{\text{XOD, OH}^\cdot, Fe^{2+}-H_2O_2} C_2H_4 + \text{products}.$$

It was earlier believed that this reaction represented a key step in the formation of the ripening hormone, ethylene in plants [4].

2) Oxidation of 1-aminocyclopropane-carboxylic acid (ACC)



It is currently believed that this reaction is the last step in the pathway of ethylene formation in plants [5, 6].

3) Ethylene formation from methionine

$CH_3S-CH_2-CH_2-CH.NH_2COOH \xrightarrow{^1O_2,\ OH^\cdot} C_2H_4$ + products (*cf.* [7, 8]).

These three reactions were studied for their abilities to be activated by TCDO as compared to other active oxygen species [9–13].

B) *Comparative reactivity of TCDO*

In the presence of a suitable substrate such as xanthine, hypoxanthine or acetaldehyde, xanthine oxidase gives rise in addition to $O_2^{\cdot-}$ and H_2O_2, to $OH^\cdot$ radical-like species which are extremely reactive and can, for example, initiate the depolymerisation of hyaluronic acid. The participation of $O_2^{\cdot-}$ and H_2O_2 in the formation of $OH^\cdot$ like radicals can be demonstrated by the inhibition of ethylene formation from KMB by SOD and catalase, respectively. In contrast to KMB, ethylene formation from MET can only be brought about by species which are thermodynamically or kinetically more reactive than the oxidant arising from the action of xanthine oxidase (Figs. 1 and 2). Such reactive species include singlet oxygen ($^1O_2$) and "crypto-$OH^\cdot$" which we proposed recently. This species is formed in the presence of both suitable one electron donor and H_2O_2 [8]. The mechanism of formation of this highly reactive oxygen species can occur *via* redox reactions catalyzed by anthraquinone as shown in the following scheme:

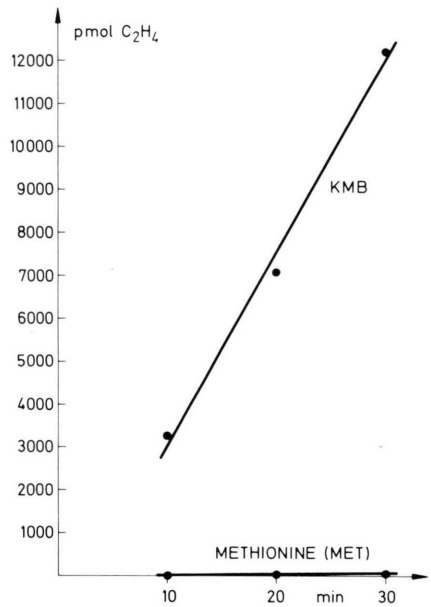


Fig. 2. Ethylene formation from S-methyl-α-ketobutyric acid (KMB) driven by xanthine oxidase and xanthine. Reaction conditions: 2 ml phosphate buffer (0.1 M, pH 7.8) contained 20 μg xanthine oxidase, 5 μmol xanthine and 10 μmol KMB.

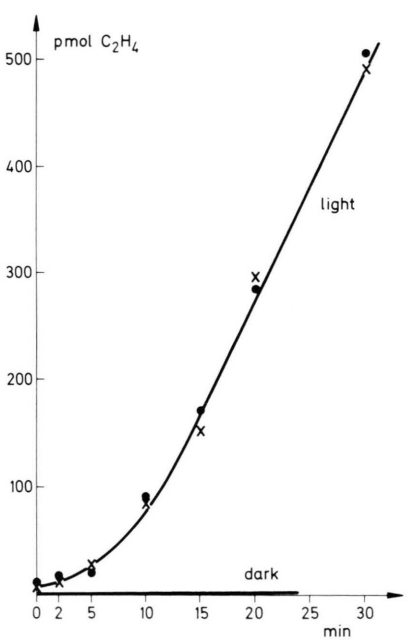


Fig. 1. Light dependent ethylene formation from methionine mediated by the singlet oxygen generator, rose bengal.
Reaction conditions: Illumination (30 klux, white light) of 2 ml phosphate buffer containing 50 μM rose bengal solution and 10 μmol methionine.
x, o = gas production in parallel vessels.

$$NADPH_2 + 2\,AQ \xrightarrow{\text{oxidoreductase}} NADP + 2\,AQH^{\cdot} \quad \text{(reduction)} \quad (1)$$

$$AQH^{\cdot} + O_2 \rightleftharpoons AQ + O_2^{\cdot-} + H^+ \quad \text{(autoxidation)} \quad (2 = 4)$$

$$2\,O_2^{\cdot-} + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad \text{(dismutation)} \quad (3)$$

$$AQ + O_2^{\cdot-} + H^+ \rightleftharpoons AQH^{\cdot} + O_2 \quad \text{(semiquinone formation)} \quad (4 = 2)$$

$$AQH^{\cdot} + H_2O_2 \longrightarrow AQ + (OH^{\cdot}) + H_2O \quad \text{(strong oxidant formation, crypto-OH}^{\cdot}\text{)}$$

This sequence of reactions is inhibited by SOD due to competition between reactions (3) and (4) since in the presence of SOD reaction (3) is approx. 10^4 faster than reaction (4). Catalase is also inhibitory by removing H_2O_2 which is required for "strong oxidant" formation according to reaction 5 (Table I). MET can also be oxidized, forming ethylene by $^1O_2$ which can be generated photochemically by the action of the pigment, rose bengal (Fig. 1). However, this reaction is not inhibited by either SOD or catalase, but is inhibited by the carotenoid, crocin which scavenges $^1O_2$ [14].

In the following report, these systems are applied in order to investigate the comparable reactivity of TCDO with respect to other active oxygen species such as $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and compound I. In addition, the activation of TCDO has been investigated with respect to metabolically relevant enzymes.

Materials and Methods

The formation of ethylene from MET, ACC, and KMB was determined by gas chromatography [7, 8], after incubation of the reaction mixture in 15 ml Fernbach vessels, fitted with serum rubber stoppers. 1 ml samples of the headspace were taken and submitted to ethylene detection by means of a flame ionisation detector. Using a 60 cm Al_2O_3-column, the retention time for ethylene was found to be approx. 28 sec.

The various enzymes and substrates used in these investigations were obtained from Boehringer, Mannheim. ACC was a gift from Drs. Lürssen and Konze, Bayer A.G. Leverkusen. Other reaction conditions are described in the legends. Oxoferin^R is commercially available from "OXOCHEMIE", Heidelberg; OF is a 3 mM, TCDO is a 150 mM aqueous solution of tetrachlorodecaoxide.

Results

1) *Oxidation of KMB*

Neither KMB nor MET are oxidized at measurable rates by TCDO alone. However, in the presence of POD, KMB is oxidized to ethylene, although MET remains unaffected. In contrast to the reaction shown in Table I the reaction with KMB is not inhibited by either catalase or SOD (Table II).

2) *Oxidation of ACC*

TCDO can oxidize ACC alone but at very low rates, which can be markedly accelerated by addition of POD. In this reaction, POD can neither be replaced by catalase, nor by Fe^{2+} or Fe^{3+} salts. Hemoglobin is able to replace POD in this reaction, whereas cytochrome c possesses about 20–25% of the activity of POD (Table III).

3) *Kinetics of ACC oxidation*

The time-course of TCDO-dependent ACC oxidation is biphasic (Fig. 3), as is the response to varying

Table I. Oxidation of methionine by reduced AQ and inhibition by SOD and catalase. Reaction conditions: 2 ml reaction volume contained; 0.1 M phosphate buffer pH 7.8; 10 μmol glucose-6-phosphate; 50 μg glucose-6-phosphate dehydrogenase; 1 μmol NADP; 0.4 μmol AQ; 10 μmol methionine; 10^{-4} M pyridoxal phosphate; 1 mg NADPH-cytochrome c-(ferredoxin)-oxidoreductase. Incubation time: 45 min at 22 °C.

Additions	pmol ethylene formed	% Inhibition
None	1800	0
100 units catalase	50	98
100 units SOD	80	95

Table II. Oxidation of S-methyl-ketobutyric acid (KMB) by TCDO: catalysis by POD. Reaction conditions: 2 ml reaction volume contained; 0.1 M phosphate buffer pH 7.8; 5 mM KMB or MET (+ 10^{-4}M pyridoxal phosphate). Incubation time; 45 min at 22 °C.

Additions	pmol ethylene/ 45 min
KMB	0
KMB + TCDO (2 μmol)	0
KMB + POD (10 U)	0
KMB + TCDO + POD	3700
KMB + TCDO + POD + 100 U catalase	3640
KMB + TCDO + POD + 100 U SOD	3800
MET + TCDO	0
MET + POD	0
MET + TCDO + POD	0

POD concentration (Fig. 4). In the presence of 10 units POD, the formation of ethylene from ACC shows a saturation curve with respect to TCDO concentration (Fig. 5). In contrast to the results with POD, the oxidation of ACC dependent upon hemoglobin shows an abrupt saturation with 2 μmol hemoglobin (Fig. 6). It was also found that the POD-catalysed reaction was strongly stimulated by the addition of Mn^{2+} (data not shown).

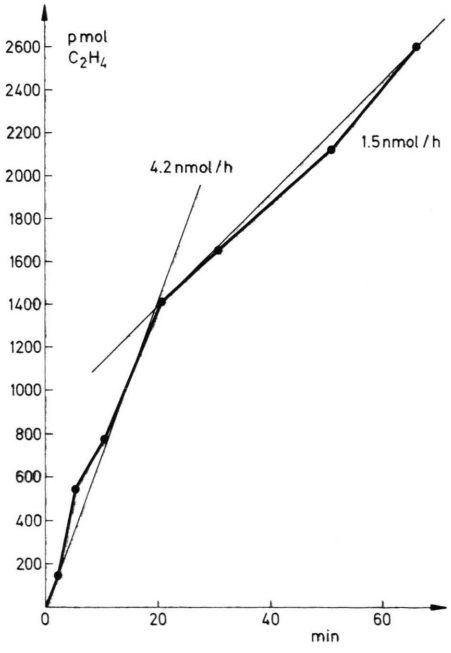


Fig. 3. Time course of ethylene formation from 1-aminocyclopropane carbonic acid (ACC).
Reaction conditions: 2 ml 0.1 M phosphate buffer pH 7.8 contained 1 mM ACC, 10 μl TCDO and 10 U POD. The reaction was conducted in 15 ml rubber-sealed Fernbach flasks at 22 °C in a water bath.

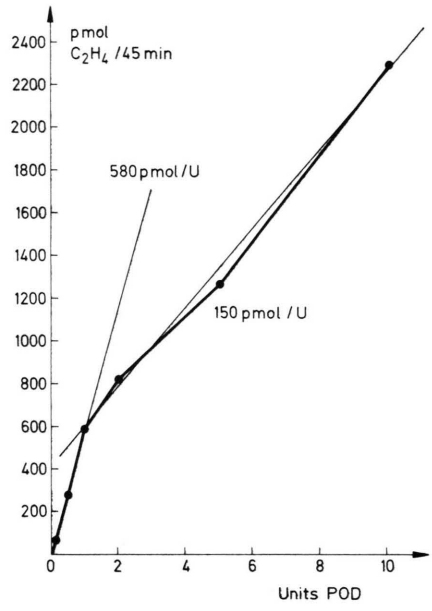


Fig. 4. Ethylene formation from 1-aminocyclopropane carbonic acid (ACC) in the presence of TCDO and varying amounts of POD.
Reaction conditions: see Fig. 3; the reaction was conducted for 45 min.

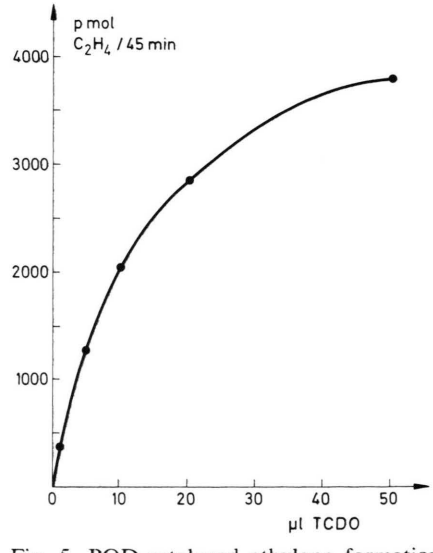


Fig. 5. POD-catalyzed ethylene formation from ACC in response to increasing TCDO-concentrations.
Reaction conditions: see Figs. 3 and 4.

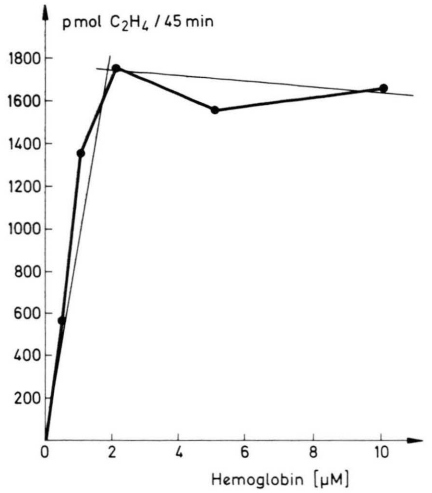


Fig. 6. Hemoglobin-catalyzed ethylene formation from ACC driven by TCDO.
Reaction conditions: see Figs. 3 and 4.

Table III. Catalysis of TCDO dependent ACC oxidation by various iron compounds. Reaction conditions: 2 ml reaction volume contained; 0.1 M phosphate buffer pH 7.8; 1 mM ACC; 2 μmol TCDO.
Incubation time; 45 min at 22 °C.

Additions	pmol ethylene/ 45 min
ACC + TCDO	20
ACC + TCDO + POD (10 U)	1668
ACC + TCDO + catalase (100 U)	16
ACC + TCDO + hemoglobin (10^{-5}M)	1525
ACC + TCDO + cytochrome c (10^{-5}M)	336
ACC + TCDO + Fe^{2+} (10^{-5}M)	24
ACC + TCDO + Fe^{3+} (10^{-5}M)	22

Discussion

The studies reported here show that TCDO does not release the OH· radical or $^1O_2$, but can give rise to an oxidant in the presence of POD or hemoglobin which appears to be similar to the POD–H_2O_2 complex comparable to the 'Klebanoff system' [15]. This latter system represents a model for the bactericidal activity of polymorphonuclear leucocytes during phagocytosis and following reactions inside these blood cells. The application of *in vitro* test reactions leading to the production of active oxygen species [12, 3] allows the following conclusions to be drawn in the case of TCDO:

1) TCDO itself is not an aggressive oxidant with comparable reactivity to the OH· radical or $^1O_2$. In contrast to these species, TCDO cannot fragment MET forming ethylene, either in the presence or absence of a catalyst (Fig. 2, Table II).

2) Similar to XOD, TCDO can oxidize KMB to ethylene (Table II). However, in contrast to the XOD reaction (see Table II in ref. [3]) these reactions are not inhibited by either catalase or SOD.

3) The reaction of TCDO under POD catalysis represents a new type of activated oxygen species which possesses certain characteristics in common with the peroxidase complex POD, NADH, Mn^{2+} (see [15–17]).

In the oxidation of ACC, hemoglobin can replace POD as the catalyst (Table III), which is also true for KMB oxidation. Similarly, hemoglobin can be replaced by either myoglobin or blood [18], however with different reaction kinetics. The different saturation kinetics of the POD and hemoglobin catalysed reactions indicate that the catalysis is dependent upon the reaction constant of the respective hemoprotein in the system. This explains why catalase showed no reaction with TCDO and is further evidence that the TCDO derived species is not identical with compound I (enzyme-H_2O_2complex). In this respect, it should be noted that both catalase and POD compound I can oxidize formate producing CO_2.

On the basis of the results reported here, it is possible to explain the clinical properties of OF in which bactericidal (bacteriostatic) effects [1] during enhanced wound healing [2] have been observed. In a following report elsewhere [18] an explanation will be presented to account for the simultaneous improvement in oxygen supply in peripheral tissue, after the application of OF.

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