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Superoxide Anion Radical Generation During the Oxidation of Various Amines by Diamine Oxidase

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Diamine oxidase (DAO) or histaminase is an enzyme which deaminates histamine and several aliphatic amines to their corresponding aldehydes. Hydrogen peroxide and ammonia are side products of this reaction. The purpose of the present work was to evaluate if determination of produced hydrogen peroxide reflects DAO activity or if intermediate formation of the superoxide radical could be a reason for lack of correspondence between oxygen uptake and hydrogen peroxide production at different pH. Superoxide radical formation was determined by cytochrome c reduction in the presence and absence of superoxide dismutase (SOD). Oxygen uptake was measured with an oxygen electrode and hydrogen peroxide production by a spectrophotometric method. At pH 6.6 there was no superoxide production, but at pH 7.4 there was some, and it increased markedly at pH 9.5. Oxygen uptake also increased with increasing pH, especially with histamine as substrate. These results lead us to suggest that the mechanism of action of DAO involves the intermediate generation of superoxide radicals.

Key words: diamine oxidase, histaminase, superoxide radical, oxygen uptake

INTRODUCTION

Cupric amine oxidases are widely distributed in nature. They perform important physiological functions, including the catabolism of endogenous and exogenous amines. Diamine oxidase (DAO) or histaminase is a cupric amine oxidase (E.C. 1.4.3.6.) and catalizes the oxidative deamination of histamine and of other amines as follows:

$$R.CH_2.CH_2.NH_2 + O_2 + H_2O \rightarrow R.CH_2.CHO + NH_3 + H_2O_2$$

DAO is characterized by the presence of a metallic cofactor (two atoms of CuII)[1,2] and a strongly electrophilic organic cofactor which reacts with carbonyl reagents.[3] Several studies have been made to elucidate the structure and the function of this carbonyl-reactive cofactor.

The first compound proposed as the prosthetic group of DAO was pyridoxal phosphate,[4] but later a tricarboxylated quinoline quinone (PQQ), was suggested.[5,6] More recent studies have demonstrated a new organic cofactor for this enzyme, 6-hydroxidopa,[7,8] with PQQ being formed as the result of ciclyzation.[9] However, DAO action mechanism is independent of the nature of the cofactor, being a transamination reaction between

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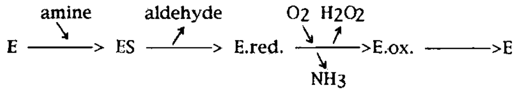
*To whom correspondence should be addressed the amine group of the substrate and the carbonyl group of the cofactor 9. Specifically, DAO acts through a substitution mechanism in which the substrate amine is initially anaerobically oxidized by the enzyme. This liberates the respective aldehyde and converts the enzyme back to its reduced form. At the next step these reduced species are reoxidized by molecular oxygen,[10,11] also forming H_2O_2 and NH_3.[12,13]

The oxidized form of the enzyme (Eox) is later converted into a more stable form (E). The second oxidation step is considered to be the rate limiting step[14]:



The step which involves reoxidation of DAO may follow a univalent pathway (equations a and b) generating superoxide radical, or a divalent pathway (equation 2) generating hydrogen peroxide:

$$E\text{-}H_2 + 2\,O_2 \rightarrow E + 2\,H^+ + 2\,O_2^{\bullet-} \qquad \text{(1a}$$

$$2\,O_2^{\bullet-} + 2\,H + \rightarrow O_2 + H_2O_2 \qquad \text{(1b}$$

$$E\text{-}H_2 + O_2 \rightarrow E + H_2O_2 \qquad \text{(2}$$

In 1966 Muraoka *et al.*[15] investigated the pathway utilized by DAO of Aspergillus niger. They performed experiments using several types of amines as substrates and oxygen or cytochrome c as electron acceptors, under aerobic and anaerobic conditions. The results obtained showed that all the amines tested preferably reduced cyt. c under anaerobiosis. Later Rotilio *et al.*[16] suggested that DAO had the capacity to generate oxygen free radicals. They also found that the enzyme reduced oxygen or cyt. c dependent on the type of amine used. Most amine oxidases use oxygen as a hydrogen acceptor, leading to the formation of hydrogen peroxide,[15] but Rotilio *et al.*[16] showed that superoxide radical was also formed during the reduction of molecular oxygen by DAO. Younes and Weser[17] confirmed this finding by showing that the DAO activity was inhibited by SOD. Copper acted in the reoxidation of the reduced form of the enzyme,[11] probably by binding oxygen in order to interact with the carbonyl cofactor[18] but no change in the redox state of copper was observed.[19] However more recently Dooley *et al.* (1991)[20] suggested that copper may change its redox state.

The purpose of this study is to specifically identify which steps in the catalytic mechanism of DAO involve the reoxidation of the enzyme by oxygen. This should allow us to determine if the electron transfer to oxygen is univalent, with superoxide formation, or bivalent, with resultant formation of hydrogen peroxide. The activity of DAO was calculated by determining the amount of hydrogen peroxide formed during the oxidative reaction. This parameter was then compared to the amount of oxygen consumed, to determine if the production of superoxide radical, as an intermediate, would exclude a parallelism between oxygen consumption and hydrogen peroxide produced. To evaluate if the relationship between hydrogen peroxide and superoxide radical was or was not influenced by pH, all experiments were performed with buffers of different pH. We selected as substrates both aromatic (benzylamine and histamine) and aliphatic amines (cadaverine and putrescine).

MATERIALS AND METHODS

DAO purified from pig's kidney was obtained from Sigma.

Two methods were used for determination of superoxide radical formation: 1) a colorimetric method based on the reduction of ferricytochrome c in the presence and in the absence of SOD[21] and 2) a manometric method for determination of oxygen consumption. For the latter a Gilson oxygraph 5/6 equipped with a Clark electrode was used in the presence and in the absence of catalase and/or

SOD.[15,16,22] For determination of hydrogen peroxide, the colorimetric method of Gordon and Peters[23] was used.

Detection of the superoxide radical by reduction of cytochrome c

The univalent reduction of oxygen to form superoxide occurring when DAO catalyzes the oxidation of various amines was determined by measuring cyt c reduction as an increase in absorbancy at 550 nm. SOD was used to determine the background rate of cyt c reduction. Measurements were made in a total volume of 1.5 ml, containing DAO 0.1 U, ferricytochrome c, 5mM, substrate, 2 mM, and buffer.

Absorbance was measured at $\lambda = 550$ nm, at 0, 15, 30 and 60 min against the respective blank in a Pharmacia-LKB-Ultrospec III. Assays with SOD 50.4 U (100 ul) added to the reaction mixture were performed simultaneously. The substrates selected were histamine, cadaverine, putrescine and benzylamine. All the assays were completed at least in quadruplicate. Buffers used were sodium phosphate 0.1 mM, pH 6.6 or 7.4 and sodium carbonate, 0.1 uM, pH 9.5.

Detection of the generation of the superoxide radical by oxygen consumption

This method is based on the inhibition of oxygen consumption by ferricytochrome c in the univalent pathway:

$$EH_2 + 2\,O_2 \rightarrow E + 2\,H^+ + 2\,O_2^{\bullet -}$$

$$2\,O_2^{\bullet -} + 2\,H^+ \rightarrow O_2 + H_2O_2$$

In the presence of cyt c there will be a decrease in oxygen consumption, since molecular oxygen increases:

$$\text{cyt c Fe (III)} + O_2^{\bullet -} \rightarrow \text{cyt c Fe (II)} + O_2$$

The assays were performed in incubation chambers subject to constant shaking. DAO 0.1 U (100 ul) and the substrate 1 mM (50 ul) were mixed in a total volume of 1.8 ml, completed by adding potassium phosphate buffer 0.1 mM pH 7.4. Oxygen consumption was measured for 30 min at 37°C. Control experiments were made adding

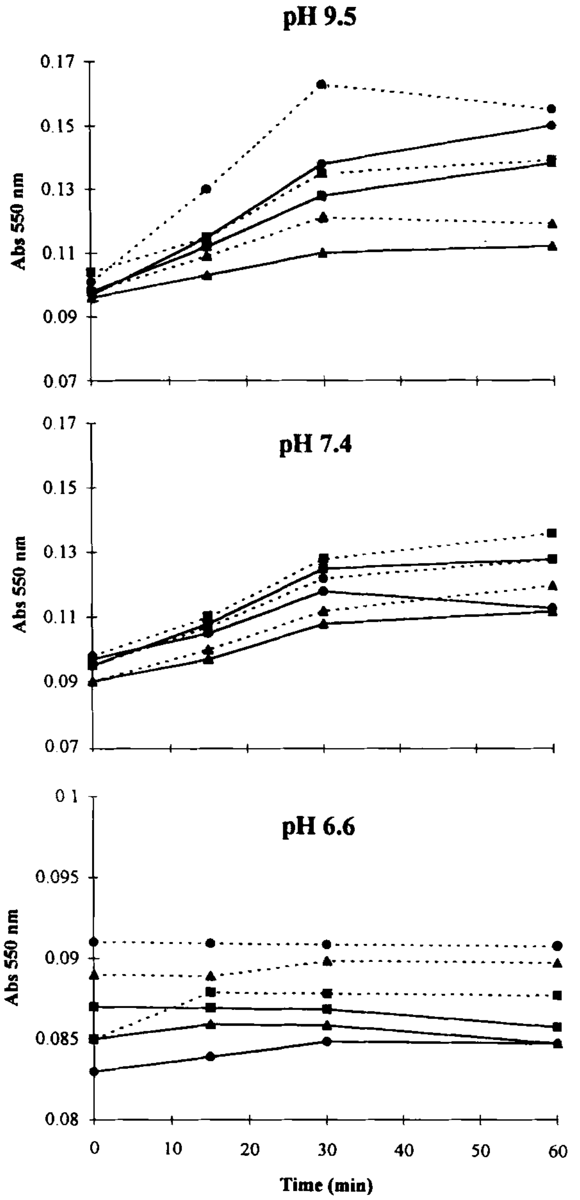


FIGURE 1 Reduction of ferricytochrome C during reactions catalyzed by DAO at pH 6.6, 7.4 and 9.5 in the presence and in the absence of SOD. DAO substrates were histamine (●), cadaverine (■) and benzylamine (▲), with SOD (——) and in its absence (– – –).

ferricytochrome c 5 mM (100 ul) in the presence and in the absence of SOD 50.4 U or of catalase 123 U (100 ul). Other buffers used were sodium phosphate 0.1mM pH 6.6 and sodium carbonate 0.1 M pH 9.5.

After calibration of the oxygraph at 0 and 100% of oxygen saturation, the enzymatic activity was determined as a function of oxygen uptake.

Determination of hydrogen peroxide

Hydrogen peroxide formed in oxidative deamination was measured using orthodianisidine in the presence of peroxidase.[23] Absorbancy units were converted to concentration units using a H_2O_2 calibration curve.

RESULTS

Spectrophotometric determination of superoxide radical formation by reduction of ferricytochrome c

Figure 1 and Table 1 show the change in absorbance at 550 nm due to reduction of cytochrome c in reactions catalyzed by DAO, using as substrates histamine, cadaverine and benzylamine, in the presence and in the absence of SOD, at pH 6.6, 7.4 and 9.5. Measurements were made at 0, 15, 30 and 60 min. Reactions were approximately linear for 30 min. An increase in absorbance was evident at pH 7.4 and especially at pH 9.5, histamine being always the best substrate. Comparing the rates of reactions at these two pHs with and without SOD, between 0 and 30 min, a reduction in the reaction rates is evident in the presence of SOD. This is more notorious at pH 9.5, but is still visible at pH 7.4.

Detection of superoxide radical formation by oxygen consumption

Table 2 presents the data obtained when oxygen consumption was measured for 30 min at pH 6.6, 7.4 and 9.5 using histamine as substrate. Also shown are the data obtained in the presence of cytochrome c, SOD and catalase. The oxygen consumption was reduced substantially in the presence of cytochrome c, especially at pH 9.5, but increased almost to control levels when SOD was

TABLE 1 Statistical analysis of the data from Figure 1, by the paired Student's test. Mean and SD of 4 determinations. Results for benzylamine and for pH 6.6 not shown, since they were not significant.

pH	amine	0 min	15 min	30 min	60 min
9.5	histamine	0.101 ± 0.002	0.130 ± 0.02	0.163 ± 0.006	0.155 ± 0.04
	" + SOD	0.097 ± 0.006	0.115 ± 0.04	0.138 ± 0.004	0.150 ± 0.008
	t	1.2649	0.93437	6.9330	0.62010
	p	ns	ns	< 0.001	ns
	cadaverine	0.104 ± 0.007	0.115 ± 0.003	0.135 ± 0.008	0.139 ± 0.004
	" + SOD	0.098 ± 0.006	0.110 ± 0.002	0.128 ± 0.002	0.130 ± 0.008
	t	1.3880	2.4361	3.4300	1.9924
	p	ns	ns	<0.01	<0.05
7.4	histamine	0.095 ± 0.003	0.107 ± 0.007	0.125 ± 0.004	0.128 ± 0.002
	" + SOD	0.097 ± 0.007	0.105 ± 0.009	0.118 ± 0.002	0.113 ± 0.006
	t	0.61538	0.36220	0.1.7537	4.9386
	p	ns	ns	ns	<0.001
	cadaverine	0.098 ± 0.003	0.110 ± 0.005	0.128 ± 0.003	0.136 ± 0.03
	" + SOD	0.095 ± 0.004	0.108 ± 0.002	0.125 ± 0.004	0.128 ± 0.007
	t	1.2930	0.76440	1.2288	2.2317
	p	ns	ns	ns	<0.05

added. Addition of catalase also restored some of the decrease in oxygen consumed in the presence of cytochrome c, but was less effective than SOD. Similar results were obtained when the other amines, cadaverine, putrescine and benzylamine were used (data not shown).

Production of hydrogen peroxide

Hydrogen peroxide production during the oxidation reaction of several amines by DAO is shown in Table 3. More H_2O_2 was produced at pH 6.6 than at pH 7.4 or 9.5 for all substrates used. However this was much more pronounced for histamine and cadaverine than for putrescine and benzylamine.

DISCUSSION

In Figure 1 we described the incubation of an amine with DAO in aerobiosis, originating the formation of an aldehyde and of superoxide:

$$\text{amine} + \text{oxygen} \rightarrow \text{aldehyde} + \text{superoxide}$$

superoxide reduces cytochrome c, increasing A_{550}:

$$\text{superoxide} + \text{cyt c Fe(III)} \rightarrow \text{oxygen} + \text{cyt c Fe(II)}$$

In the presence of SOD, part of the superoxide disappears due to dismutation. As a result, the increase in A_{550} is smaller:

$$\text{amine} + O_2 \longrightarrow \text{aldehyde} + O_2 \begin{cases} \longrightarrow O_2 + H_2O_2 & \text{(SOD)} \\ \longrightarrow O_2 & \text{(cyt c).} \end{cases}$$

We find in Figure 1 that the generation of superoxide ion is important at pH 9.5, less at pH 7.4 and undetectable at acidic pH. These results are similar for several amines, but more evident with histamine.

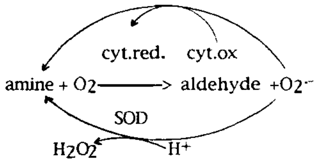
In Table 2 the study is similar, but now we measure oxygen consumed, not reduced cytochrome c. Only the experiments using histamine as substrate are presented, since they are the most evident.

a – as we described earlier, the reaction of an amine with oxygen, in the presence of DAO, generates superoxide and so takes oxygen up:

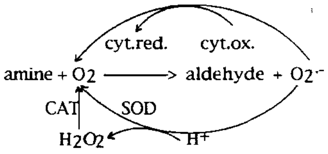
$$\text{amine} + O_2 \rightarrow \text{aldehyde} + O_2^{\bullet -}$$

$$\text{cyt c Fe(III)} + O_2^{\bullet -} \rightarrow \text{cyt c Fe(II)} + O_2$$

b – adding SOD, part of the superoxide dismutates, forming H_2O_2 and O_2. So there is less superoxide to react with cyt c:



c – catalase does not act in this system unless it is contaminated with SOD. In this case it decreases the oxygen consumption:



Finally in Table 3 it is demonstrated that hydrogen peroxide production is maximal at acidic pH, when there is no generation of superoxide radicals. This agrees entirely with the expected facts:

$$\text{amine} + O_2 \rightarrow \text{aldehyde} + H_2O_2 \text{ pH low}$$

$$\text{amine} + O_2 \rightarrow \text{aldehyde} + O_2^{\bullet -} \text{ pH high}$$

As we see, some results do not agree with the expected facts, namely the effect of SOD and of catalase. This leads us to believe that there may be an unspecific reaction of undetermined nature of SOD on the reaction catalyzed by DAO.

In what concerns catalase, it is probably contaminated with SOD and this explains why the reaction is similar, although less intense, to that of SOD. Although several schemes have been suggested for the catalytic mechanism of cupric

TABLE 2 Influence of pH in the formation of $O_2^{\bullet -}$ calculated from the O_2 consumption, when histamine was used as the substrate. Results expressed in umols O_2 consumed during 30 min. Means + SD. (CS: complete system)

	a pH 6.6		b pH 7.4		c pH 9.5	
	umols O_2	%O_2	umols O_2	%O_2	umols O_2	%O_2
1. CS	81.9 ± 6.8 n = 5	38.2	94.2 ± 2.9 n = 7	44.0	155.2 ± 5.6 n = 9	72.5
2. CS + cyt c	59.9 ± 4.5 n = 5	28.0	57.8 ± 1.1 n = 5	27.0	100.0 ± 1.2 n = 5	45.0
3. CS + cyt c + SOD	75.5 ± 3.8 n = 5	35.3	91.0 ± 3.0 n = 7	42.5	139.8 ± 3.7 n = 5	65.3
4. CS + cyt c + CAT	65.3 ± 5.1 n = 5	30.5	76.0 ± 7.6 n = 5	35.5	123.2 ± 5.3 n = 5	57.6
1/2	t = 5.5087 p<0.001		t = 26.150 p<0.001		t = 21.306 p<0.001	
1/3	t = 1.8336 ns		t = 2.0183 p<0.05		t = 44.134 p<0.001	
1/4	t = 4.3740 p<0.001		t = 5.8649 p<0.001		t = 10.369 p<0.001	
2/3	t = 5.8934 p<0.001		t = 23.194 p<0.001		t = 28.850 p<0.001	
2/4	t = 1.7614 ns		t = 5.3230 p<0.001		t = 9.5416 p<0.001	
3/4	t = 3.6111 p<0.001		t = 4.5174 p<0.001		t = 5.7076 p<0.001	
1. . .t = 4.3232 a/b p<0.001	2. . .t = 1.0259 a/b ns		3. . .t = 7.9030 a/b p<0.001		4. . .t = 2.6300 a/b. . .p<0.02	
1. . .t = 21.760 a/c p<0.001	2. . .t = 19.140 a/c p<0.001		3. . .t = 27.173 a/c p<0.001		4. . .t = 17.669 a/c p<0.001	
1. . .t = 25.963 b/c p<0.001	2. . .t = 57.750 b/c p<0.001		3. . .t = 25.188 b/c p<0.001		4. . . .t = 11.438 b/c p<0.001	

amine oxidases, only two mechanisms involve the formation of an intermediate free radical.[24] The catalytic mechanism of DAO is generally described as a transamination reaction resulting in the formation of a Schiff's base. This model has persisted since the identification of pyridoxal phosphate as cofactor for the enzyme.[9] But, according to Pedersen, the quinone cofactors may undergo the addition of primary amines in the 1,4 position by a different mechanism. One of the most controversial steps in the mechanism of DAO is the second step, aerobic, which is initiated by the formation of a binary complex with oxygen. Dooley and Coolbaugh[24] suggested two hypotheses for the transfer of one electron during reoxidation of the enzyme: a) the superoxide anion is dismutated, generating H_2O_2 or b) the transfer occurs first to molecular oxygen, being later transferred to the copper present in the active center of the enzyme, without a change in the redox status of the metal ion.[25]

In 1988 Paz *et al.*,[26] after identifying PQQ as the prosthetic group of pig's kidney DAO, demonstrated that putrescine, when oxidized by DAO, generated the superoxide radical. PQQ in reduced form, which would react with oxygen in a one electron transfer, generating superoxide, detected by NBT reduction. However Gallop *et al.*[16] found that the aerobic reduction of NBT might result from the direct transfer of two electrons from the

TABLE 3 Concentration of hydrogen peroxide (umol) formed in the reaction of oxidation of several amines by DAO during 30 min at 37° C, at pH 6.6, 7.4 and 9.5. Mean and SD of 4 experiments each.

	a – pH 6.6	b – pH 7.4	c – pH 9.5
1. histamine	20.03 ± 0.95	13.20 ± 0.42	1.46 ± 0.13
2. cadaverine	9.40 ± 0.79	6.48 ± 0.31	1.39 ± 0.08
3. putrescine	8.88 ± 0.59	3.18 ± 0.18	4.28 ± 0.87
4. benzylamine	5.52 ± 0.098	3.60 ± 0.83	3.34 ± 063
1/2	t = 17.207 p<0.001	t = 0.16712 ns	t = 0.91717 ns
1/3	t = 19.941 p<0.001	t = 43.856 p<0.001	t = 6.4116 p<0.001
1/4	t = 30.386 p<0.001	t = 20.640 p<0.001	t = 5.8450 p<0.001
2/3	t = 1.0548 ns	t = 18.412 p<0.001	t = 6.6157 p<0.001
2/4	t = 9.7481 p<0.001	t = 6.5017 p<0.001	t = 6.1411 p<0.001
3/4	t = 11.236 p<0.001	t = 0.98900 ns	t = 1.7502 ns
1. . .t = 13.151 a/b p<0.001	2. . .t = 6.8815 a/b p<0.001	3. . .t = 18.481 a/b p<0.001	4. . .t = 4.5945 a/b p<0.001
1. . .t = 38.733 a/c p<0.001	2. . .t = 20.175 a/c p<0.001	3. . .t = 8.7520 a/c p<0.001	4. . .t = 6.8384 a/c p<0.001
1. . .t = 53.405 b/c p<0.001	2. . .t = 31.797 b/c p<0.001	3. . .t = 2.4763 b/c p<0.02	4. . .t = 0.4990 b/c ns

reduced form of the enzyme to oxygen, without forming superoxide as an intermediate. More recently Shah *et al.*[27] suggested that during oxygen reduction, a one electron transfer could occur, generating superoxide and/or hydroxyl radical, possibly due to a Fenton type reaction between hydrogen peroxide and copper. Thus, in the scientific literature several hypotheses exist regarding the nature of the intermediate produced during the catalytic cycle of DAO.

Our results employing the reduction of ferricytochrome c agree with the reduction of oxygen by a univalent pathway. The increase in absorbance determined corresponded to the reduction of cytochrome c by superoxide generated during the catalytic cycle of DAO, since it was inhibited by SOD. This was confirmed by our experiments measuring oxygen consumption. Oxygen consumption may follow two different pathways, one of univalent reduction with formation of superoxide and another one of bivalent reduction, with formation of hydrogen peroxide. In saturating concentrations ferricytochrome c may inhibit part of the oxygen consumption by the univalent pathway but not by the bivalent pathway. Our results on the percentage of oxygen consumption showed that, in the presence of ferricytochrome c, there was a decrease in the percentage of oxygen consumed.

Besides, if SOD is also present, it can, by catalysing a dismutation reaction, compete with ferricytochrome c for the superoxide ion. It can therefore tend to overcome the effect of cytochrome c on the oxygen consumption. If we add catalase the percentage of consumption decreases because the H_2O_2 produced in the bivalent reduction is transformed by catalase.[22]

DAO has different affinities for various amines. Histamine is a good substrate at pH 6.6 while cadaverine has been described as a better substrate at pH 7.4.[28] Our results do not confirm this finding. Fridovich (1970)[22] established that the fraction of the total flux of electrons which traversed the univalent pathway to oxygen was increased by raising the pH.

Regarding the concentration of hydrogen peroxide formed, our results show that the highest production occurs at an acidic pH, contrary to that for superoxide. Rotilio *et al.*[16] showed that the aerobic reduction of cytochrome c by DAO was dependent on pH, with a higher stability of superoxide at an alkaline pH, in which the generation of the superoxide radical is favoured. Although the most common mechanism for DAO activity includes transfer of 2 electrons, it is important to mention the superoxide anion as an intermediate of the catalytic cycle of this enzyme, since, in accordance with our results, there must be transfer of one electron to oxygen, even at acidic pH, which is probably dismutated in the presence of hydrogen ions existing in solution. Another mechanism may be related to the formation of a substrate-cofactor radical, as described by Janes *et al.*[8] when 6-hydroxydopa was identified as cofactor of DAO. Copper has been implicated in the reoxidation of the substrate-reduced enzyme, by generating a Cu(I)-semiquinone state.[20]

This reactivity of DAO to form superoxide anions in vivo is controlled by scavenger systems, like SOD and catalase, but may induce pathological situations when they are inoperative. Amine oxidases are highly active enzymes in many organs, especially in the central nervous system. Their ability to produce oxygen free radicals may be a contributory factor in the induction of oxidative stress and tissue damage in the CNS.

As a final conclusion, we may state that our data confirms previous data from other authors which suggested the generation of the superoxide radical during diamine oxidase activity.

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