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Determination of catalase activity in samples treated with [$ZnCl_2$(isopropylamine)$_2$]: a novel zinc complex that slows down the decay in activity of catalase extracts

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Reaction of $ZnCl_2$ with an excess of isopropylamine ligand in water gives [$ZnCl_2$(isopropylamine)$_2$]. This novel zinc complex has been characterized by elemental analysis and ^{1}H-NMR. Interestingly, we have found that [$ZnCl_2$(isopropylamine)$_2$] markedly retards the decay in activity of catalase extracts. In fact, catalase extracts (0.075 mg of protein ml^{-1}) may retain more than 80% of their initial enzymatic activity within the first 10 hours of incubation with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$] while the activity of control extracts decreases to less than 50% of the initial value. Moreover, after 24 hours of treatment with [$ZnCl_2$(isopropylamine)$_2$] under the above-mentioned conditions, 70% of the initial enzymatic activity is still retained by catalase extracts while the activity of control untreated extracts drops to 17% of the initial value. On the other hand, our results show that after incubation of catalase extracts with [$ZnCl_2$(isopropylamine)$_2$] the supernatants obtained by centrifugation of the extracts contain a higher amount of active catalase than the supernatants of control untreated catalase extracts. Moreover, [$ZnCl_2$(isopropylamine)$_2$] induces precipitation of a large amount of contaminant proteins present in the catalase extracts. Altogether, these data indicate that treatment of catalase extracts with [$ZnCl_2$(isopropylamine)$_2$] both increases the pureness of catalase solutions and slows down the decay in catalase activity. We believe that [$ZnCl_2$(isopropylamine)$_2$] may be useful as a stabilizing agent for enzyme activity assays with crude catalase extracts.

1 Introduction

Reactive oxygen species such as the superoxide radical, hydroxyl radical, and hydrogen peroxide, are formed during reduction of molecular oxygen to water. The potential of these species to damage proteins, lipids, and nucleic acids requires the existence of antioxidants as well as enzymes.[1] Two families of enzymes capable of degradation of hydrogen peroxide are present in all aerobic organisms, namely catalases and peroxidases.[2] Lack or malfunction of catalases may lead to severe defects such as apoptotic cell death,[3] increased susceptibility to thermal injury,[3] high rates of mutations,[4] and, in higher organisms, inflammation.[5]

Catalase (EC 1.11.1.6, hydrogen peroxide: hydrogen peroxide oxidoreductase) is present in almost every aerobic organism and serves to protect cells from the toxic effects of H_2O_2 by catalyzing the reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (1)$$

Catalases are ubiquitous enzymes which have been isolated from a broad range of prokaryotic and eukaryotic organisms. Most catalases described so far are tetramers with molecular weights ranging from 220 to 270 kDa with each subunit containing a protoheme as prosthetic group.[1] The structures of seven heme-containing catalases have been determined at almost atomic resolution.[6–11] Despite all the structural and biochemical information available, there is a major limitation in the high-resolution structural results from catalase complexes because these data are difficult to obtain.[12] Beef liver catalase (BLCase) is a tetramer (MW = 230,000 kDa), roughly dumbbell-shaped, with molecular 222 symmetry. The structure determination of BLCase catalase crystals has been reported at several degrees of resolution.[13,14]

Metal compounds can affect the activity of enzymes and enzyme systems within the cell. In fact, the short term exposure of animals to Ni or Hg, as well as long-term Fe treatment of animals results in stimulation of membrane lipid peroxidation.[15,16] In addition, Cu and Zn can inhibit *in vitro* aminopirine-*N*-demethylase and at high concentrations, these two metals produce the same effect on aniline hydroxilase activity as well.[17] On the other hand, ethanol and Triton X-100 (a non-ionic detergent) have been reported as two substances that potentiate catalase activity in several crude tissue extracts.[18] Ethanol increases the observable catalase levels by decomposing Complex II, which is an inactive complex of catalase with H_2O_2 formed spontaneously in catalase extracts. Triton X-100 also increases observable catalase levels in tissue extracts, but through solubilization of particle-bound catalase. In this paper we report the synthesis and characterization of the novel zinc complex [$ZnCl_2$(isopropylamine)$_2$]. This complex substantially delays the decay in activity shown by catalase extracts. Moreover, the data reported here suggest that [$ZnCl_2$(isopropylamine)$_2$] increases the purity of crude catalase preparations through precipitation of contaminant proteins present in the catalase extracts.

2 Results

2.1 Characterization of [$ZnCl_2$(isopropylamine)$_2$]

The reaction of $ZnCl_2$ with an excess of isopropylamine in aqueous solution and subsequent slow evaporation of the

Table 1 Catalase activity values (mean ± SD) in BLCase extracts (0.075 mg of protein ml^{-1}) incubated with several concentrations of [$ZnCl_2$-(isopropylamine)$_2$], $ZnCl_2$ and isopropylamine for 24 hours at 37 °C. SD = standard deviation

		Enzyme activity/units mg $protein^{-1}$	
	[Compound]/μM	1 hour	24 hours
Control catalase		2183 ± 104	371 ± 16
[$ZnCl_2$(isopropylamine)$_2$]	0.05	2084 ± 106	262 ± 20
	0.10	2096 ± 102	1404 ± 70
	0.25	2181 ± 110	720 ± 35
$ZnCl_2$	0.05	2160 ± 108	360 ± 18
	0.10	2174 ± 104	369 ± 22
	0.25	2087 ± 112	372 ± 17
Isopropylamine	0.05	2190 ± 109	370 ± 20
	0.10	2164 ± 96	367 ± 14
	0.25	2092 ± 103	355 ± 17

solvent yielded the [$ZnCl_2$(isopropylamine)$_2$] complex. The microanalytical data (see Experimental section) are consistent with the proposed empirical formula $ZnCl_2C_6H_{18}N_2$ and in agreement with the structure $ZnCl_2(L)_2$. The ^{1}H-RMN chemical shifts (δ ppm) of the isopropylamine ligand and the [$ZnCl_2$(isopropylamine)$_2$] compound are also given in the Experimental section. The amine group signal is the most affected due to coordination of the zinc atom to the nitrogen of the NH_2 group. The differences in the chemical shift of the methyl groups of the [$ZnCl_2$(isopropylamine)$_2$] complex and the isopropylamine ligand are in the order of 0.5 and 0.4 ppm, being lower than those of the amine group due to the fact that the methyl group is far away from the coordination site. It is most likely that, as previously reported for other Zn(II) complexes with N donors and bulky ligands,[19–23] the Zn(II) atom of [$ZnCl_2$(isopropylamine)$_2$] adopts a distorted tetrahedral conformation (Fig. 1).

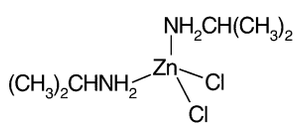

Fig. 1 Proposed structure for the [$ZnCl_2$(isopropylamine)$_2$] complex.

2.2 Protective effect of [$ZnCl_2$(isopropylamine)$_2$] on the activity of catalase extracts

Among the first row of transition metals, Zn is second only to Fe in terms of abundance and importance in biological processes.[24] In addition, it has been previously reported that catalase activity may be affected in liver homogenates from rats supplemented with metal compounds including zinc compounds.[25] Based on these grounds, we wanted to study the effect on activity of catalase extracts of [$ZnCl_2$(isopropylamine)$_2$], $ZnCl_2$, isopropylamine as well as other zinc and related metallic compounds such as $ZnSO_4$, $Zn(NO_3)_2$, ZnO, $CuCl_2$, $CdCl_2$ and $CrCl_3$. Table 1 shows the catalase activities of beef liver catalase (BLCase) extracts incubated with [$ZnCl_2$(isopropylamine)$_2$], $ZnCl_2$ or isopropylamine. It may be observed that after 24 hours of incubation at 37 °C of control untreated BLCase extracts the enzyme activity decayed to 17% of the initial activity present in the extract. Interestingly, however, incubation of BLCase extracts for 24 hours with [$ZnCl_2$(isopropylamine)$_2$] at concentrations of 0.10 and 0.25 μM induced a much lower decrease in catalase activity. Thus, after 24 hours of incubation at 37 °C of BLCase extracts with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$], the activity was 67% of the initial activity present in the extract. At 0.25 μM [$ZnCl_2$(isopropylamine)$_2$], BLCase activity was 33% of the initial activity. However, neither $ZnCl_2$, nor isopropylamine nor even the other metallic compounds had any observable effect on the catalase activity of BLCase extracts in the range of concentrations and periods of incubation tested. Similar results were obtained with bovine or mouse liver catalase extracts or when the incubations were carried at room temperature (data not shown).

Fig. 2 shows the variation of catalase activity as a function of the period of incubation at 37 °C of BLCase extracts with [$ZnCl_2$(isopropylamine)$_2$] at its optimum concentration of 10 μM. We used ethanol and Triton X-100 as positive controls because they are two well-known enhancers of catalase activity in crude tissue extracts. It may be observed that catalase activity in control untreated BLCase extracts decayed as a function of time from 0 to 48 hours of incubation at 37 °C. The highest decay in activity (from 80 to 50% of the initial activity) was observed in the interval between 5 and 9 hours of incubation. It is interesting to point out that BLCase activity was only 17% of the initial activity after 24 hours of incubation at 37 °C. Moreover, after 48 hours of incubation, the activity of control BLCase extracts was only 5% of the initial value. In contrast, incubation at 37 °C of BLCase extracts with 0.10 μM [$ZnCl_2$-(isopropylamine)$_2$] resulted in a delay in the decrease of activity of catalase. Thus, within the first 10 hours of incubation with [$ZnCl_2$(isopropylamine)$_2$] the BLCase extracts retained more than 80% of the initial activity. Moreover, after 24 hours of incubation of BLCase extracts with [$ZnCl_2$(isopropylamine)$_2$] the activity was about 70% of the initial value. After 48 hours of incubation with the zinc complex, the catalase extracts still maintained 33% of their initial activity. As shown in Fig. 2, it is also interesting to note that [$ZnCl_2$(isopropylamine)$_2$] did not

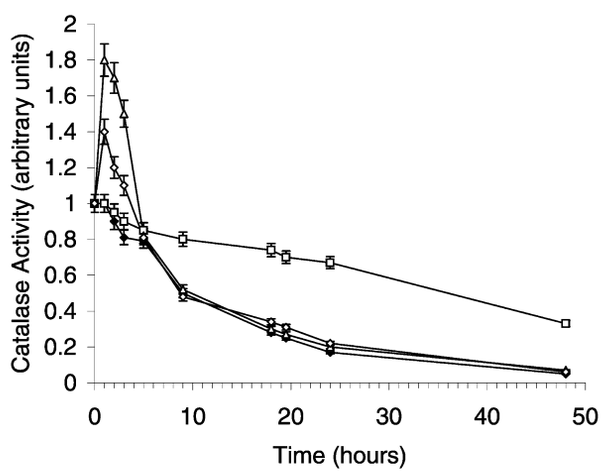

Fig. 2 Time-dependent changes in catalase activity of beef liver catalase (BLCase) extracts (0.075 mg of protein ml^{-1}) incubated at 37 °C with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$] (□), 0.10 μM ethanol (△) or 1% (v/v) Triton X-100 (◇) and in control BLCase extracts (◆).

increase catalase activity above the value of activity found in freshly prepared control BLCase extracts. In contrast, both ethanol and Triton X-100 increased catalase activity in catalase extracts at short periods of incubation (interval between 1 and 2.5 hours). Thus, Fig. 2 shows that after 1 hour of incubation at 37 °C of BLCase extracts with 0.10 μM ethanol or 1% (v/v) of Triton X-100, catalase activity was 1.8-times and 1.4-times, respectively, higher than that of control extracts. However, after 5 hours of incubation of BLCase extracts with either ethanol or Triton X-100, catalase activity decayed in a way similar to that found in control catalase extracts. These results indicate that either ethanol or Triton X-100 enhance catalase activity in BLCase extracts at short periods of incubation. In contrast, $[ZnCl_2(isopropylamine)_2]$ induces a protective effect on catalase activity of BLCase extracts over a wide range of periods of incubation but does not produce potentiation of catalase activity. Similar results were found with bovine or mouse liver catalase extracts (data not shown). On the other hand, we did not observe any effect on the activity of crystalline bovine or beef liver catalases incubated with $[ZnCl_2(isopropylamine)_2]$, ethanol or Triton X-100 over a wide range of concentrations and periods of incubation (data not shown).

2.3 Metal binding to crystalline bovine liver catalase

Zn(II) usually functions as an electrophilic cofactor in catalytic mechanisms or stabilizes peptide or protein structure. In both cases, Zn(II) can appear as a single bound ion or in a cluster with other metal ions.[26] Because $[ZnCl_2(isopropylamine)_2]$ does not have any observable effect on the activity of crystalline beef or bovine liver catalase it is likely that $[ZnCl_2(isopropylamine)_2]$ does not bind to the enzyme. In order to prove the lack of zinc coordination to catalase, crystalline bovine liver catalase dissolved in 50 mM buffer phosphate, pH 7.0, at a concentration of 0.05 μM was incubated with 0.25 μM $[ZnCl_2(isopropylamine)_2]$ or K_2PtCl_4 for several periods of time (molar ratio of Zn or Pt : catalase of 5 : 1). Afterwards, the samples were dialysed overnight and Zn or Pt content was determined by TXRF. Potassium tetrachloroplatinate was used as a positive control of metal binding to catalase since it is known that Pt from $PtCl_4^{2-}$ coordinates to this enzyme.[13] As expected, the data shown in Fig. 3 indicate that $[ZnCl_2(isopropylamine)_2]$ does not

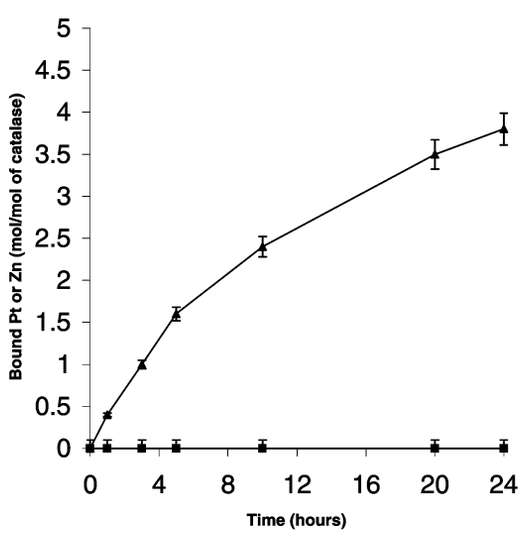

Fig. 3 Time course of metal binding (0.25 μM solution of metallic complex) to crystalline bovine liver catalase (0.05 μM) as measured by TXRF. (▲) K_2PtCl_4, (■) $[ZnCl_2(isopropylamine)_2]$. The input molar ratio of metal : nucleotide was 5 : 1.

bind to bovine liver catalase. Similar data were obtained with crystalline beef liver catalase (data not shown). These results are in agreement with the fact that, so far, no Zn-binding sites have been found in catalases from several sources.[27] In contrast, Pt from K_2PtCl_4 binds to catalase in a time-dependent way. After 24 hours of incubation, 76% of the input Pt atoms from K_2PtCl_4 were bound to bovine liver catalase because the ratio of mol of bound Pt/mol of catalase was 3.8. This value is in agreement with the fact that the enzyme is a tetramer with 1 Pt binding site per monomer.[28]

Table 2 Optical density values obtained at 300 nm for BLCase extracts (0.075 mg of protein ml^{-1}) incubated for 1 hour or 24 hours with 0.10 μM $[ZnCl_2(isopropylamine)_2]$, $ZnCl_2$ or ethanol and 1% (v/v) of Triton X-100. SD = standard deviation

	$OD_{300\ nm}$ (mean ±SD)	
	1 hour	24 hours
Control catalase	0.012 ± 0.07	0.064 ± 0.06
$[ZnCl_2(isopropylamine)_2]$	0.010 ± 0.04	0.126 ± 0.04
$ZnCl_2$	0.012 ± 0.05	0.066 ± 0.04
Ethanol	0.011 ± 0.04	0.067 ± 0.05
Triton X-100	0.012 ± 0.05	0.060 ± 0.03

2.4 Effect of $[ZnCl_2(isopropylamine)_2]$ on catalase solutions from liver extracts

As shown above, $[ZnCl_2(isopropylamine)_2]$ does not increase the activity of crystalline catalase and, moreover, $[ZnCl_2(isopropylamine)_2]$ does not bind to the enzyme. Thus, it is reasonable to assume that preservation of catalase activity induced by $[ZnCl_2(isopropylamine)_2]$ in catalase extracts must be due to an indirect effect on catalase. In fact, during the course of our experiments on catalase activity with crude extracts we observed that the turbidity of the solutions of the extracts incubated with $[ZnCl_2(isopropylamine)_2]$ increased in a time-dependent way.

Table 2 shows the values of optical density at 300 nm obtained in control BLCase extracts and in BLCase extracts incubated with 0.10 μM $[ZnCl_2(isopropylamine)_2]$, $ZnCl_2$ and ethanol and with 1% (v/v) of Triton X-100 for 1 hour and 24 hours at 37 °C. It may be observed that after 24 hours of incubation, the turbidity in BLCase extracts and in BLCase extracts treated with $ZnCl_2$, ethanol or Triton X-100 increased 5-times relative to a period of incubation of 1 hour. However, the turbidity of BLCase extracts incubated with $[ZnCl_2(isopropylamine)_2]$ for 24 hours increased 10-times relative to the turbidity of the control extracts incubated for 1 hour. These results indicate that after 24 hours of incubation, the precipitation of proteic components from BLCase extracts treated with $[ZnCl_2(isopropylamine)_2]$ is 2-times higher than that of control BLCase extracts and BLCase extracts incubated with $ZnCl_2$, ethanol or Triton X-100.

Fig. 4A shows the results obtained when aliquots of supernatants or resuspended pellets obtained from BLCase extracts incubated for 5 hours at 37 °C with 0.10 μM $[ZnCl_2(isopropylamine)_2]$ were subjected to SDS-12% PAGE under denaturing conditions. It may be observed that the amount of protein precipitate is 2-times higher in the resuspended pellets of BLCase extracts treated with $[ZnCl_2(isopropylamine)_2]$ (lane 9) than in the resuspended pellets of control BLCase extracts (lane 6). Moreover, it is interesting to note that the band corresponding to BLCase monomers (MW ≈ 57.500 kDa, see control lane 2 for allocation) is principally found in the supernatants of BLCase extracts treated with $[ZnCl_2(isopropylamine)_2]$ and also in control BLCase (lanes 8 and 5, respectively). Fig. 4B shows the results obtained when aliquots of supernatants obtained from BLCase extracts treated with 0.10 μM $[ZnCl_2(isopropylamine)_2]$ and incubated for 24 hours at 37 °C were subjected to SDS-12% PAGE under denaturing conditions. It may be observed that the band corresponding to BLCase monomers (MW ≈ 57.500 kDa, see control lane 2) has a higher intensity in

Table 3 Zinc concentrations (ng ml^{-1}) measured by TXRF in supernatants and precipitates of BLCase extracts (0.075 mg of protein ml^{-1}) incubated for 5 hours or 24 hours with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$] equivalent to a concentration of Zn of 6.54 ng ml^{-1}. The data are expressed as the mean ± standard deviation of four determinations

	[Zn]/ng ml^{-1}	
	5 hours	24 hours
Supernatants	2.60 ± 0.13	0.62 ± 0.02
Precipitates	3.90 ± 0.15	5.85 ± 0.35

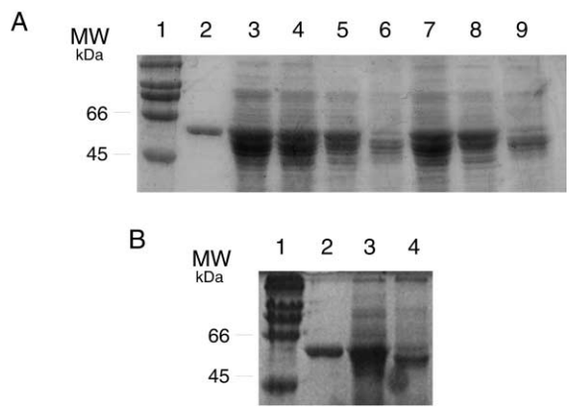

Fig. 4 (A) SDS-12% polyacrylamide gel electrophoresis under denaturing conditions of aliquots of supernatants or resuspended pellets obtained from BLCase extracts (0.075 mg of protein ml^{-1}) incubated for 5 hours at 37 °C with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$]. Lane 1: molecular weight marker; lane 2: control crystalline BLCase; lane 3: control BLCase extract incubated at 24 °C; lane 4: control BLCase extract incubated at 37 °C; lane 5: supernatant of control BLCase extract incubated at 37 °C; lane 6: pellet of control BLCase extract incubated at 37 °C; lane 7: BLCase extract incubated at 37 °C with [$ZnCl_2$(isopropylamine)$_2$]; lane 8; supernatant of BLCase extract incubated at 37 °C with [$ZnCl_2$(isopropylamine)$_2$]; lane 9: pellet of BLCase extract incubated at 37 °C with [$ZnCl_2$(isopropylamine)$_2$]. (B) SDS-12% polyacrylamide gel electrophoresis under denaturing conditions of aliquots of supernatants obtained from BLCase extracts incubated for 24 hours at 37 °C with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$]. Lane 1: molecular weight marker; lane 2: control crystalline BLCase; lane 3: supernatant of BLCase extract incubated at 37 °C with [$ZnCl_2$(isopropylamine)$_2$]; lane 4: supernatant of control BLCase extract incubated at 37 °C.

the supernatants obtained from the BLCase extracts incubated with [$ZnCl_2$(isopropylamine)$_2$] (lane 3) than in those obtained from control extracts (lane 4). Interestingly, we found that catalase activity in the supernatants from BLCase extracts incubated with [$ZnCl_2$(isopropylamine)$_2$] for 24 hours at 37 °C (Fig. 4B, lane 3) was 70% of the initial activity present in the BLCase extracts. In contrast, the activity found in supernatants of control BLCase extracts incubated for 24 hours at 37 °C (Fig. 4B, lane 4) was only 10% of the activity present in the extracts. In addition, the resuspended pellets from control BLCase extracts and from BLCase extracts incubated with [$ZnCl_2$(isopropylamine)$_2$] did not exhibit catalase activity (data not shown).

TXRF measurements of zinc in supernatants as well as in precipitates of BLCase extracts incubated with [$ZnCl_2$(isopropylamine)$_2$] revealed that the concentration of zinc present in the precipitates was significantly higher than that present in the supernatants. In fact, Table 3 shows that after 5 hours of incubation at 37 °C of BLCase extracts with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$] which corresponds to a zinc input of 6.54 ng ml^{-1}, 60% of the zinc input was found in the precipitate and 40% in the supernatant. Moreover, after 24 hours of incubation at 37 °C of BLCase extracts with 0.10 μM [$ZnCl_2$(isopropylamine)$_2$], 90% of the zinc input was found in the precipitate and only 10% in the supernatant. Similar data were obtained with bovine or mouse liver catalase extracts (data not shown). These results suggest that [$ZnCl_2$(isopropylamine)$_2$] favours the precipitation of a large amount of the contaminant proteins present in crude catalase extracts but without inducing precipitation of catalase.

3 Discussion

We report here the synthesis and characterization of the novel compound [$ZnCl_2$(isopropylamine)$_2$]. This zinc complex has been characterized by elemental analysis, TXRF and ^{1}H-NMR. TXRF measurements show that the Zn : Cl ratio is 1 : 2. In addition, ^{1}H-NMR data indicate that there is no formation of hydroxo complexes. We think that the lack of hydroxo complexes may be due to the steric effect of the ligand. As in the case of other Zn(II) complexes with N donors and bulky ligands, we propose that most likely the Zn(II) centre of [$ZnCl_2$(isopropylamine)$_2$] adopts a distorted tetrahedral geometry.[19–23]

From the results of the present study it is clear that [$ZnCl_2$(isopropylamine)$_2$] substantially protects catalase activity in catalase extracts. In contrast, neither $ZnCl_2$ nor the isopropylamine ligand are able to induce this effect on the activity of catalase extracts. In addition, other zinc compounds such as $ZnSO_4$ and $Zn(NO_3)_2$ do not have any effect on the activity of catalase extracts. These results are in agreement with previously reported data indicating that the catalase activity in liver homogenates of rats supplemented with $ZnSO_4$ is similar to that of liver homogenates of control rats.[25]

It has been previously reported that incubation of crude tissue extracts with ethanol or Triton X-100 can result in marked increases in catalase activity. Ethanol increases the amount of active catalase molecules by a redox mechanism in which inactive Complex II with oxidation state of Fe(IV) is transformed to active Ferricatalase with oxidation state of Fe(III).[29] Triton X-100 also increases the number of active catalase molecules in tissue extracts but through a different mechanism that involves solubilization of particle-bound catalase.[18] However, no increase in catalase activity was observed when crystalline catalase was treated with ethanol nor Triton X-100.[18] Thus, in experiments with catalase extracts in which total catalase levels are desired, the use of ethanol plus Triton X-100 assures catalase solubilization and prevents catalase inactivation within the first five hours after preparation of the catalase samples. However, from a survey of the literature, we have found that the use of ethanol or Triton X-100 preparatory to the determination of catalase activity in crude tissue extracts is far from general practice. Moreover, the combination of these two enhancers of catalase activity is rare in evaluations of catalase activity in tissue extracts.

We report here that [$ZnCl_2$(isopropylamine)$_2$] markedly retards the decay in catalase activity shown by catalase extracts incubated at 37 °C (or room temperature) over a wide range of periods of incubation (up to 48 hours). However, at short periods of incubation and in contrast with ethanol or Triton X-100, [$ZnCl_2$(isopropylamine)$_2$] does not increase catalase activity above that shown in control extracts. Thus, we think that, contrary to ethanol or Triton X-100, [$ZnCl_2$(isopropylamine)$_2$] does not increase the number of active catalase molecules in solution. It is known that Zn(II) does not have biological redox activity but, instead, it usually works as an electrophilic cofactor in catalytic mechanisms or stabilizes protein structure.[19,26] The TXRF data reported here show that there is no zinc coordination to catalase after incubation of crystalline catalase with [$ZnCl_2$(isopropylamine)$_2$]. Although some sort of reversible association between catalase and [$ZnCl_2$(isopropylamine)$_2$] might occur, we have not observed by means of circular dichroism spectroscopy changes in the secondary or tertiary structures of catalase in the presence

of [$ZnCl_2$(isopropylamine)$_2$] (data not shown). Moreover, our data indicate that [$ZnCl_2$(isopropylamine)$_2$] does not vary the activity of crystalline catalase. Therefore, it is most likely that preservation of catalase activity in catalase extracts by [$ZnCl_2$-(isopropylamine)$_2$] is related to an indirect effect of this novel zinc complex on catalase. In fact, the spectrophotometric and electrophoretic data reported herein show that after treatment of catalase extracts with [$ZnCl_2$(isopropylamine)$_2$], a large amount of the proteins initially present in the crude catalase extract precipitates. However, [$ZnCl_2$(isopropylamine)$_2$] does not seem to favour precipitation of catalase because the supernatants obtained from extracts treated for 24 hours with [$ZnCl_2$(isopropylamine)$_2$] contain a higher amount of catalase molecules than the supernatants of control untreated extracts. Moreover, TXRF determinations of zinc content in catalase extracts treated for 24 hours with [$ZnCl_2$(isopropylamine)$_2$] show that most zinc is found in the precipitate of contaminant proteins. Therefore, the results suggest that incubation of [$ZnCl_2$(isopropylamine)$_2$] with catalase extracts induces precipitation of a large amount of proteins but without significantly affecting the number of catalase molecules in solution. We believe that the precipitation of contaminant proteins induced by [$ZnCl_2$(isopropylamine)$_2$] in the extracts might favour better access of H_2O_2 to catalase in solution.

Altogether, the results reported here indicate that [$ZnCl_2$(isopropylamine)$_2$] may be used in catalase extracts as a stabilizing agent, especially when the enzymatic activity is not going to be determined within the first hours after preparation of catalase samples. On the other hand, we think that the use of [$ZnCl_2$(isopropylamine)$_2$] as a stabilizing agent of catalase activity in catalase extracts may be complementary to that of enhancers of catalase activity such as ethanol and Triton X-100.

4 Experimental

4.1 Materials

Isopropylamine, $ZnCl_2$, $ZnSO_4$, $Zn(NO_3)_2$, $CuCl_2$, $CdCl_2$ and $CrCl_3$ were purchased from Aldrich Chemical Co. Ethanol absolute and Triton X-100 were obtained from Merck. Cisplatin, [*cis*-diamminedichloroplatinum(II)], was obtained from Sigma Co. Potassium tetrachloroplatinate was a gift from Johnson Matthey. Stock solutions of the compounds (1 mg ml^{-1}) were prepared in distilled water. Bovine and mouse liver catalase extracts as well as crystalline bovine liver catalase were purchased from Sigma Co. Beef liver catalase extracts and crystalline beef liver catalase were obtained from ICN Pharmaceuticals, Inc. The specific activities ranged from 900 units mg^{-1} of protein to 2.310 units mg^{-1} of protein for crude liver catalase extracts and 46.500 units mg^{-1} of protein for crystalline catalase, respectively. One unit of specific activity is defined as the amount of enzyme which decomposes 1.0 μmol of H_2O_2 per minute at pH 7.0 and 25 °C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM. Catalase extracts were dissolved in 50 mM buffer phosphate, pH 7.0, at a protein concentration of 0.075 mg ml^{-1}. Crystalline catalase was dissolved in 50 mM buffer phosphate pH 7.0 at a concentration of 0.05 μM. H_2SO_4 was supplied by Merck Co. $KMnO_4$ was purchased from PROLABO. H_2O_2 (30% w/v) was supplied by FORET S.A.

4.2 Methods

NMR spectra were recorded on a Brucker AMX-300 (300 Mhz) spectrometer in $CDCl_3$ solution. Elemental analyses for C, N, H were performed on a Perkin Elmer 2400 Series II microanalyzer. Elemental analyses for Cl were performed using Schöniger techniques with some modifications.[30]

4.2.1 Synthesis of [$ZnCl_2$(isopropylamine)$_2$]. 0.3 g of $ZnCl_2$ (2.2 mmol) was dissolved in 4 ml of distilled water (Millipore quality). Subsequently, an excess of isopropylamine (6 ml, 70 mmol) was added to the solution under stirring at room temperature. A white precipitate appeared when the pH of the suspension was changed from 4 to 12. The suspension was filtered and then allow to dry at room temperature. Afterwards, the white crude product was dissolved in methanol and precipitated with Et_2O. The resultant white solid was recrystallised from methanol. Finally, the solid was redisolved in acetone and precipitated with Et_2O.

[$ZnCl_2$(isopropylamine)$_2$] complex. Yield: 60%. (Found: C 28.68; H 7.57; N 11.25, Cl 28.29. Calc.: C 28.32; H 7.13; N 11.01, Cl 28.40%). ^{1}H NMR (300 MHz, $CDCl_3$): δ ($2CH_3$) = 1.36 d (6H), δ (CH) = 3.46 sp (1H), δ (NH_2) = 5.31 b.s. (2H). Isopropylamine was purchased from Aldrich Chemical and used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ ($2CH_3$) = 0.89 d (6H), δ (CH) = 2.92 sp (1H), δ (NH_2) = 0.95 b.s. (2H).

4.2.2 Enzyme activity assays. Catalase activity was determined by a redox procedure.[31] A solution of 10 ml of 0.4% of H_2O_2 was used as enzyme substrate. After 5 minutes of incubation of 0.4% of H_2O_2 with 1 ml of catalase extracts (0.075 mg ml^{-1}) or 0.05 μM of crystalline catalase, the H_2O_2 decomposition reaction was stopped with 10 ml of 1M H_2SO_4. Catalase decomposes H_2O_2 according to eqn. 2:

$$5H_2O_2 + 2KMnO_4 + 4H_2SO_4 \longrightarrow 2MnSO_4 + 2KHSO_4 + 5O_2 + 8H_2O \quad (2)$$

where $KMnO_4$ acts as oxidant and H_2O_2 acts as reductant. $KMnO_4$ was added dropwise with a burette to the sample until a change to a pale pink colour was observed. This colour change indicated that all the remaining H_2O_2 had been oxidized by $KMnO_4$. The initial amount of hydrogen peroxide was titred in a sample without enzyme. The amount of H_2O_2 decomposed in each sample containing enzyme was calculated by subtracting the initial amount of H_2O_2 in the sample without enzyme from the amount of H_2O_2 obtained in each sample containing catalase. The results were expressed as percentage change in enzyme activity assuming that under the above-mentioned experimental conditions decomposition of H_2O_2 by catalase follows first-order kinetics.[18] Catalase activity was also determined spectrophotometrically at 480 nm.[18]

4.2.3 Effect of [$ZnCl_2$(isopropylamine)$_2$] on catalase activity. 1 mg ml^{-1} stock solutions of [$ZnCl_2$(isopropylamine)$_2$], $ZnCl_2$, $ZnSO_4$, $Zn(NO_3)_2$, $CuCl_2$, $CdCl_2$, $CrCl_3$, isopropylamine and ethanol were prepared in distilled water. These compounds were incubated with catalase extracts (0.075 mg of protein m^{-1}) or crystalline catalase (0.05 μM) in 50 mM buffer phosphate pH 7.0 by addition of the desired amount of compound to the enzyme solution to obtain final compound concentrations of 0.05, 0.10 and 0.25 μM. Triton X-100, as received from the supplier, was added directly from the bottle to the enzyme buffered-solution to obtain a final concentration of 1% (v/v). After several periods of incubation (1, 2.5, 5, 9, 18, 19.5, 24 and 48 h) at 37 °C, the enzyme activity was determined by addition of H_2O_2 to the samples to a concentration of 0.4%. The catalase reaction was allowed to proceed for 5 min at room temperature and then was stopped with 1M H_2SO_4. Under these conditions the catalase reaction follows linear first-order kinetics.[18]

4.2.4 Removal of contaminating metals from working solutions. Contaminating metals were removed from plasticware and buffers by routine treatment with Chelex-100 (Bio-Rad laboratories) as previously described.[32]

4.2.5 Total reflection X-ray fluorescence analysis. The analysis by TXRF was performed using a Seifert Extra-II

spectrometer (Seifert, Ahrensburg, Germany). TXRF determinations were carried out according to a procedure previously reported.[32,33] Briefly, crystalline catalase (0.05 μM) in 50 mM buffer phosphate, pH 7.0, was incubated with 0.25 μM [$ZnCl_2$(isopropylamine)$_2$], $ZnCl_2$ and potassium tetrachloroplatinate (K_2PtCl_4) for several periods of time (1, 3, 5, 10, 20 and 24 h). The samples were dyalized overnight against 50 mM buffer phosphate, pH 7.0. A 100 μl aliquot of control catalase (0.05 μM) in 50 mM buffer phosphate, pH 7.0, was introduced into a 2 ml test tube. This solution was standardised with 100 ng ml^{-1} of Vanadium [Merck (Darmstadt,Germany) ICP Vanadium standard solution]. Afterwards, the sample was introduced into a high-purity nitrogen flow concentrator at a temperature of 70 °C until the volume was reduced five times. An aliquot of 5 μl was then taken, deposited on a previously clean quartz-made reflector and dried on a ceramic plate at a temperature of 50 °C. The entire process was done in a laminate flow chamber (Model A-100). The samples were analysed following the X-ray Molybdenum line under working conditions of 50 kV and 20 mA with a live-time of 1000 s and a dead time of 35%. Spectra were recorded between 0 and 20 keV. The following 15 elements were simultaneously analysed: P, S, K, Ca, V, Fe, Cu, Zn, As, Br, Rb, Sr, Ni, Mn and Pt, in order to obtain a correct deconvolution of profiles associated with the general spectrum. The Zn and Pt lines were used for Zn and Pt quantification, respectively. The analytical sensitivity of the TXRF measurements was 0.3 to 22.4 ng of Zn or Pt in a solution volume of 100 μl, with repeatability between 2 and 8% (3 repeats).

A [$ZnCl_2$(isopropylamine)$_2$] solution in ethanol was analyzed by TXRF, the Zn : Cl mass ratio for this sample was 1 : 2. This TXRF determination was done in a Rich&Seifert, Germany Model Extra II with the source of the X-ray from a Moαline like, Si(Li) detector and 157 eV resolution for KαMn.

4.2.6 Pt and Zn determinations. To determine the amount of Pt or Zn bound to crystalline catalase, the amount of Pt or Zn present in the control samples was subtracted from the amount of Pt or Zn present in the dialysis membranes containing catalase. The dialysis was performed in plastic containers.

4.2.7 Turbidity measurements. Turbidity of catalase extracts (0.075 mg of protein ml^{-1}) and of catalase extracts (0.075 mg of protein ml^{-1}) incubated with 0.10 μM solutions of the compounds was measured at 300 nm as described previously [32] in a Shimadzu UV-Vis spectrophotometer UV-240 Graphtcord.

4.2.8 Protein content. The amount of protein in catalase extracts and crystalline BLCase was determined by the Bradford method.[34]

4.2.9 Polyacrylamide gel electrophoresis of catalase extracts. [$ZnCl_2$(isopropylamine)$_2$] was incubated with crude catalase extracts (0.075 mg of protein ml^{-1}) in 50 mM buffer phosphate, pH 7.0. The final [$ZnCl_2$(isopropylamine)$_2$] concentration was 0.10 μM. After 24 and 48 hours of incubation, the samples were centrifuged at 12.000 rpm. Aliquots of 10 μl from the supernatants were subjected to SDS-12% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions.[35] Pellets were resuspended in 50 mM buffer phosphate, pH 7.0, and aliquots of 10 μl were also subjected to SDS-12% PAGE under denaturing conditions. Zn content in aliquots of supernatants and pellets was determined by TXRF.[32,33]

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