Activation of the NADPH-dependent H_2O_2 -generating system in pig thyroid particulate fraction by limited proteolysis and Zn^{2+} treatment

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The NADPH-dependent H_2O_2 -generating system in a pig thyroid particulate fraction requires micromolar concentrations of Ca^{2+} for activity. The H_2O_2 generator could be Ca^{2+} -desensitized (i.e. made fully active in the absence of Ca^{2+}) by limited proteolysis with α -chymotrypsin or by treatment with ZnCl_2 . The Zn^{2+} effect was temperature- and dose-dependent with an apparent half-maximum concentration of 0.15 mM at 40 °C. Ca^{2+} desensitization was not reversed by adding the Zn^{2+} chelators, 1,10-phenanthroline and EGTA, but about one-third of the Ca^{2+} -sensitivity was recovered after addition of 10 mM-dithiothreitol. The proteolysed enzyme and the Zn^{2+} -treated enzyme had different K_m values for NADPH. The Zn^{2+} effect did not seem to involve proteolysis or membrane fusion. These results indicate that Ca^{2+} regulation occurs via an autoinhibitory domain or inhibitory protein component of the H_2O_2 -generator system. Its inhibitory effect may be removed by proteolysis or conformational changes, making the catalytic site accessible to the substrate NADPH and/or enabling electrons to be transferred from NADPH to O_2 .

INTRODUCTION

Thyroid hormones are synthesized on the apical surface of the follicular cells during or after fusion of the exocytosis vesicles with the apical membrane. The final steps, iodination of thyroglobulin tyrosine residues and their coupling to form the hormones, are catalysed by thyroid peroxidase in the presence of H_2O_2 . It has been shown that H_2O_2 generation, which is regulated in intact cells by thyrotropin [1,2], plays a key role in thyroid hormone synthesis. The H_2O_2 concentration controls the relative rate of iodination and coupling [3]. H_2O_2 generation is closely linked to the generation of NADPH by the pentose phosphate pathway [4]. A variety of physiological and pharmacological agents that activate H_2O_2 formation also stimulate the pentose phosphate pathway [5]. Consequently, although several enzyme systems [6–8] have been proposed for H_2O_2 production, NADPH oxidase (EC 1.11.1.2) seems to play a significant role in thyroid H_2O_2 formation. Histological studies on isolated rat [9] and pig [10] thyroid follicles and on rat thyroid [11] indicate that NADPH-dependent H_2O_2 formation occurs on the apical cell surface. The rate of H_2O_2 generation by resting pig open follicles incubated in a Ca^{2+} -containing medium increases when the Ca^{2+} ionophore A23187 is added [10]. The stimulation of H_2O_2 generation by thyrotropin on calf slices [12] or open pig thyroid follicles [13] is not mimicked by dibutyl cyclic AMP [10] or forskolin [12,13]. Moreover, in the human thyroid, thyrotropin activates generation of inositol phosphates and this effect is not mimicked by forskolin [14]; inositol 1,4,5-trisphosphate activates Ca^{2+} release from endoplasmic reticulum [15–17]. Physiological concentrations of thyrotropin are found to produce acute increases in intracellular Ca^{2+} concentration in normal pig thyroid cells in culture [18]. Thus Ca^{2+} could well be a second messenger of thyrotropin action in these H_2O_2 -generating systems.

Studies carried out in our laboratory showed that a pig thyroid particulate fraction contains an NADPH-dependent H_2O_2 -generating system that requires submicromolar concentrations of Ca^{2+} for its activity [19]. This Ca^{2+} regulation is reversible and

seems to occur by a direct action of Ca^{2+} on the particulate enzyme. The Ca^{2+} -activated NADPH-dependent H_2O_2 generator is associated with thyroid plasma membranes [20,21]. We previously proposed that the activation of the NADPH-dependent H_2O_2 generator was modulated by a protein factor associated with the system. An inhibitor protein was extracted from the NADPH-dependent H_2O_2 generator in the presence of high Ca^{2+} concentrations (1 mM) and the generator's activity became Ca^{2+} -independent [22]. The inhibitor protein could be returned to give a Ca^{2+} -dependent system. However, the Ca^{2+} -induced release of this inhibitor depended greatly on the origin of the pig thyroid gland, for unknown reasons. The present study was carried out to explore further the activation of the NADPH-dependent H_2O_2 generator. Limited proteolysis of the pig thyroid particulate fraction or treatment with Zn^{2+} in the presence of proteinase inhibitors gave an H_2O_2 generator that was fully active in the absence of Ca^{2+} .

Regulation by Ca^{2+} is thought to proceed via an autoinhibitory domain or an inhibitory protein component of the NADPH-dependent H_2O_2 generator. Under the conditions used in this study, the activation by limited proteolysis or Zn^{2+} treatment was found to be largely irreversible.

MATERIALS AND METHODS

NADPH, freeze-dried horseradish peroxidase (grade I; EC 1.11.1.7), DNAase I from bovine pancreas (grade II), aprotinin from bovine lung and bestatin were obtained from Boehringer Mannheim; phenylmethanesulphonyl fluoride, leupeptin (synthetic, hemisulphate), Tos-Lys- CH_2Cl -treated α -chymotrypsin from bovine pancreas (type VII), α_2 -macroglobulin, phosphoramidon, benzoyl-L-tyrosyl-*p*-aminobenzoic acid, 1,10-phenanthroline, BSA and CHAPS were purchased from Sigma Chemical Co. Scopletin was from Serva.

Thyroid particulate fraction was prepared as previously described [19] and stored frozen at -20°C . The preparation of plasma membranes was adapted from Nakamura *et al.* [20] as previously described [21].

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Treatment of particulate fraction with α -chymotrypsin

Frozen particulate fraction was pelleted by centrifugation at 8000 *g* for 15 min at 4 °C. The pellet was resuspended (15 mg/ml) in 20 mM-Tris/HCl buffer, pH 8, containing 2 mM- CaCl_2 , 1 mM- MgCl_2 , 0.1 mg of phenylmethanesulphonyl fluoride-treated DNAase I/ml and 10 mM-CHAPS. Portions were incubated with Tos-Lys- CH_2Cl -treated α -chymotrypsin (0–30 *N*-benzoyl-L-tyrosine ethyl ester units/ml) for 15 min at 30 °C. Proteolysis was stopped by adding 1.5 mM-phenylmethanesulphonyl fluoride and the suspension was diluted with 50 mM-sodium phosphate buffer, pH 7.2, containing 0.25 M-sucrose and 1 mM-EGTA (buffer A) before assay of NADPH-dependent H_2O_2 formation.

Treatment of particulate fraction with ZnCl_2

Fresh or frozen particulate fraction, in 50 mM-sodium phosphate buffer, pH 7.2, containing 0.25 M-sucrose, 1 mM- MgCl_2 , 10 μM -FAD, 20 μg of leupeptin/ml, 10 μg of aprotinin/ml, 0.5 mM-phenylmethanesulphonyl fluoride and 0.1 mg of phenylmethanesulphonyl fluoride-treated DNAase I/ml was incubated with various concentrations of ZnCl_2 for 30 min at 40 °C. The reaction was stopped by placing the tubes in crushed ice, and particulate material was collected by centrifugation at 8000 *g* for 15 min at 4 °C. The pellet was washed once with 15 vol. of buffer A. When Zn^{2+} treatment was performed in the presence of 5 mM- or 10 mM-dithiothreitol, the pellet was washed with 30 vol. of buffer.

The final pellet was resuspended in buffer A and the concentration adjusted to 4 mg of protein/ml.

Preparation of membrane fraction from Zn^{2+} -treated particulate fraction

Particulate fraction (10 mg) was incubated in 12 ml of 50 mM-sodium phosphate buffer, pH 7.2, containing 2 mM- MgCl_2 and 0.1 mg of phenylmethanesulphonyl fluoride-treated DNAase I/ml for 20 min at 4 °C and then centrifuged at 4200 *g* for 30 min. The pellet was resuspended in 15 ml of 50 mM-sodium phosphate buffer, pH 7.2, containing 0.25 M-sucrose, 2 mM- MgCl_2 and 3.6 ml of a stock Percoll solution (1 vol. of 2.5 M-sucrose + 9 vol. of Percoll) and centrifuged in a Sorvall SS 34 rotor at 12000 rev./min (17300 *g*) for 30 min. The Percoll gradient was collected as 16 fractions. Protein concentration and NADPH-dependent H_2O_2 formation were measured. Density-marker beads were used to calibrate the Percoll gradients.

Assay of NADPH-dependent H_2O_2 -generating activity

Portions of Zn^{2+} -treated particulate fraction were incubated at 30 °C in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.25 M-sucrose, 2 mM-EGTA and 1 mM- NaN_3 , with or without 2.2 mM- CaCl_2 . The reaction was started by adding 0.1 mM-NADPH, and 100 μl samples were collected at time intervals and mixed with 10 μl of 1.2 M-HCl to stop the reaction by destroying the remaining NADPH.

Initial rates of H_2O_2 formation were determined on eight samples from each assay. The amount of H_2O_2 in each sample was determined in a Perkin-Elmer MPF 43A spectrofluorimeter as previously described [19].

The initial rate of H_2O_2 formation, measured in the presence of EGTA, is expressed as a percentage of the initial rate measured in the presence of Ca^{2+} (percentage Ca^{2+} desensitization).

Protein concentration

Particulate material was denatured with 1.2 M-NaOH and protein concentration was determined by the method of Bradford [23], with BSA as standard.

All experiments were performed on several thyroid particulate fractions and were run at least in triplicate.

RESULTS AND DISCUSSION

Limited proteolysis of particulate fraction or plasma membranes by α -chymotrypsin

The effect of limited proteolysis on Ca^{2+} regulation of the H_2O_2 -generator system was studied. Fig. 1 shows that, under our assay conditions, α -chymotrypsin treatment of the particulate fraction completely activated the system in the absence of Ca^{2+} with no significant loss of specific activity. The low concentrations of α -chymotrypsin used (3–10 *N*-benzoyl-L-tyrosine ethyl ester units) increased specific activity by about 40 %. Similar results were obtained by treating plasma membranes with α -chymotrypsin (results not shown).

The loss of Ca^{2+} action confirms that this cation has a direct action on the H_2O_2 -generator system, as previously suggested [21]. These results also show that activation of the H_2O_2 -generator system can proceed by the proteolytic removal of an auto-inhibitory domain or an inhibitory protein component of the H_2O_2 -generator complex.

Zn^{2+} treatment of particulate fraction

Treating the particulate fraction with ZnCl_2 produced an NADPH-dependent H_2O_2 generator that was active in the absence of Ca^{2+} . This Ca^{2+} desensitization by Zn^{2+} was a function of the treatment temperature. Fig. 2 shows that the Zn^{2+} effect appeared from 30 °C and reached a maximum at 40 °C.

The specific activity of the NADPH-dependent H_2O_2 -generator system remained constant during the 30 min Zn^{2+} treatment, up to a temperature of 45 °C, but decreased rapidly thereafter (results not shown). The kinetics of the Ca^{2+} desensitization at 40 °C showed that the Zn^{2+} effect was slow and required at least 15 min (Fig. 2 inset). Zn^{2+} treatment was therefore carried out routinely for 30 min at 40 °C. Only the particulate fraction was treated, as the H_2O_2 generator in the membrane fraction was denatured at 40 °C under the experimental conditions used.

Ca^{2+} desensitization of the particulate fraction depended on the Zn^{2+} concentration (Fig. 3): 50 % desensitization was ob-

Fig. 1. Ca^{2+} desensitization of NADPH-dependent H_2O_2 generation by α -chymotrypsin

Particulate fraction was incubated with α -chymotrypsin as described in the Materials and methods section. H_2O_2 generation was measured in the presence (●) and in the absence (○) of Ca^{2+} . Inset: percentage Ca^{2+} desensitization as a function of α -chymotrypsin concentration. The initial rate of H_2O_2 formation, measured in the presence of EGTA, is expressed as a percentage of the initial rate measured in the presence of Ca^{2+} .

Fig. 2. Effect of temperature on the Ca^{2+} desensitization of NADPH-dependent H_2O_2 generator by ZnCl_2

Particulate fractions were incubated for 30 min with (■) and without (□) 1 mM- ZnCl_2 at the indicated temperatures. Inset: kinetics of Ca^{2+} desensitization at 40 °C by 1 mM- ZnCl_2 .

Fig. 3. Ca^{2+} desensitization of the NADPH-dependent H_2O_2 generator as a function of ZnCl_2 concentration

Particulate fractions were treated with ZnCl_2 for 30 min at 40 °C as described in the Materials and methods section. Inset: irreversible effect of ZnCl_2 on Ca^{2+} desensitization as a function of 1,10-phenanthroline concentration. Particulate fractions were treated with 1 mM- ZnCl_2 at 40 °C. After 25 min of incubation, 1,10-phenanthroline was added and the incubation was continued for 5 min at 40 °C.

tained with 0.15 mM- ZnCl_2 . In some experiments, Zn^{2+} chelator was added to investigate the reversibility of the phenomenon. As this medium contained 1 mM- MgCl_2 to protect the particulate fraction against thermal denaturation, 1,10-phenanthroline or EGTA was used to chelate the Zn^{2+} , instead of EDTA. Particulate fraction was incubated with 0.15 mM- ZnCl_2 for 25 min; 1,10-phenanthroline (up to 5 mM) was added and the incubation was continued for 5 min at 40 °C. The specific activity of the H_2O_2 -generator system remained constant during this short incubation with 1,10-phenanthroline. The inset of Fig. 3 shows that Ca^{2+} desensitization was not altered by concentrations of 1,10-phenanthroline from 1 to 5 mM. However, this Zn^{2+} chelator is

hydrophobic and could form a stable ternary complex with Zn^{2+} and the H_2O_2 -generator system [24]. Incubation of particulate fraction with 5 mM-1,10-phenanthroline with or without 1 mM- ZnCl_2 for 30 min resulted in the loss of about 80 % of the enzyme activity, suggesting the irreversible formation of inactive complexes.

The addition of EGTA (5–15 mM) after 20 min of Zn^{2+} treatment did not alter the Ca^{2+} desensitization (results not shown), indicating that Zn^{2+} triggered an irreversible effect, such as molecular reorganization of membranes during fusion, reactions involving Zn^{2+} -dependent proteinases or other covalent modification of the H_2O_2 -generator system.

Uncontrolled H_2O_2 production causes severe damage to the cells, and its formation must be closely associated with cellular events, and, in the thyroid gland, with exocytosis. The thyroid peroxidase and H_2O_2 -generating system, which are not functional in the exocytosis vesicles, become active when these vesicles fuse with the apical plasma membrane [25]. The molecular reorganization of the membrane during the fusion process may allow not only the functional positioning of the thyroid peroxidase activity and H_2O_2 -generator system [26], but may also induce a conformational change in the H_2O_2 -generator system to initiate H_2O_2 formation. Bivalent cations can mediate the fusion of liposomes or biological membranes by interacting with negatively charged phospholipids [27]. Zn^{2+} induces fusion of phospholipid vesicles at concentrations of 0.2–1 mM [28].

The densities of the membranes prepared from Zn^{2+} -treated particulate fraction were examined by Percoll density-gradient centrifugation to determine whether there was any correlation between Zn^{2+} -induced Ca^{2+} desensitization and membrane fusion. Only one membrane fraction (density 1.055) that catalysed H_2O_2 formation was isolated from a 10 % Ca^{2+} -desensitized particulate fraction (treated with 2 mM- ZnCl_2 for 30 min at 4 °C). A particulate fraction that was 80 % Ca^{2+} desensitized (2 mM- ZnCl_2 for 30 min at 40 °C) gave three membrane fractions, with densities of 1.055, 1.098 and over 1.1 (i.e. at the bottom of the tube). H_2O_2 formation was catalysed only by the membrane fraction with a density of 1.055. Thus the Ca^{2+} desensitization of the H_2O_2 -generator system by Zn^{2+} does not result from membrane fusion.

Particulate fractions were routinely treated with Zn^{2+} in the presence of the serine proteinase and cysteine-proteinase inhibitors phenylmethanesulphonyl fluoride, leupeptin and aprotinin. The effects of several other proteinase inhibitors were tested to determine whether the Ca^{2+} desensitization induced by Zn^{2+} could result from the activation of a metalloproteinase (Table 1). α -Macroglobulin, which inhibits the activity of all classes of proteinase (acid, serine, cysteine and metal) towards high- M_r endopeptidases [29], phosphoramidon, which inhibits Zn^{2+} peptidases [30], and bestatin, an aminopeptidase *N* inhibitor, all did not block Ca^{2+} desensitization. Lastly *N*-benzoyl-L-tyrosyl-p-aminobenzoate, a synthetic substrate of phosphoramidon-insensitive metalloendopeptidases [30], had no effect.

Zn^{2+} is also said to inhibit phosphatases [31], but the inhibitors of phosphatase activities, β -glycerophosphate plus fluoride, did not mimic the Zn^{2+} effect on the H_2O_2 -generating system. Thus Zn^{2+} treatment does not involve inhibition of dephosphorylation.

Ca^{2+} desensitization could also result from modification of chemically labile groups, such as thiol groups. Any oxidation during the Zn^{2+} treatment was prevented by performing all incubations under anaerobic conditions (argon atmosphere). These conditions did not affect Ca^{2+} desensitization (results not shown).

Dithiothreitol (10 mM) blocked the Ca^{2+} -desensitizing effect of Zn^{2+} treatment at 40 °C when it was added at zero time. Dithiothreitol is a metal-ion-complexing agent [32], and the con-

Table 1. Effect of proteinase and phosphatase inhibitors or substrates on the percentage Ca^{2+} desensitization of particulate fraction

Treatment	Ca^{2+} desensitization (%)
1 mM- ZnCl_2	75
1 mM- ZnCl_2 + 1 unit/ml of α -macroglobulin	75
1 mM- ZnCl_2 + 2–10 μM -phosphoramidon	76
1 mM- ZnCl_2 + 0.5–2 mM-benzoyl- <i>p</i> -aminobenzene	72
1 mM- ZnCl_2 + 0.1 mg/ml of bestatin	75
40 mM- NaF + 20 mM- β -glycerophosphate	10

centration used was sufficient to prevent the Zn^{2+} effect under our conditions.

However, when 10 mM-dithiothreitol was added after 20 min of Zn^{2+} treatment, and the incubation was continued for 10 min at 40 °C, the particulate fraction recovered about one-third of its Ca^{2+} -sensitivity. This partial reversibility was tentatively increased by washing the preparation with 1 mM-EGTA and leaving it for 24 h in 5 mM-dithiothreitol at 0 °C. The percentage of desensitization reached a minimum plateau of 50 % (Fig. 4).

Zn^{2+} interacts with proteins mainly by co-ordination binding with cysteine and/or histidine residues [33]. The Zn^{2+} effect observed was temperature-dependent, and heating, which destabilized membrane structures, could make residues available for Zn^{2+} chelation. Several studies have shown that Zn^{2+} binding mainly favours interactions between protein, subunits or different protein domains [34–36]. Metal-ion-binding domains help stabilize protein structure, and Zn^{2+} might induce and stabilize conformational changes in the H_2O_2 -generator system that would disrupt the association of an autoinhibitory domain or protein component with the active site, making it functional or accessible to the substrate NADPH. This type of mechanism has been proposed to explain the activation of protein kinase C by phorbol esters and diacylglycerol. These activators are thought to bind to a cysteine-rich region, and consequently remove the pseudo-substrate from the active centre [37–39].

The reversibility of the Zn^{2+} effect indicates that some Zn^{2+} chelators, 1,10-phenanthroline and EGTA, do not reverse the Zn^{2+} effect, whereas dithiothreitol can partially reverse the phenomenon. The autoinhibitory domain or protein component could be partially re-instated after the removal of Zn^{2+} only in the presence of dithiothreitol, which might act as a protector.

The Zn^{2+} -treated enzyme had the same V_{max} for NADPH as substrate when H_2O_2 formation was measured in the presence and in the absence of Ca^{2+} (Fig. 5), corresponding to 100 % of Ca^{2+} desensitization for an infinite NADPH concentration. However, the K_m values were different: K_m was $20 \pm 2 \mu\text{M}$ in the presence of Ca^{2+} and $50 \pm 5 \mu\text{M}$ in its absence. Similar K_m values ($25 \pm 5 \mu\text{M}$) were obtained for proteolysed enzyme in the presence and in the absence of Ca^{2+} . These kinetic data indicate that NADPH was more accessible to the active centre of the proteolysed enzyme than to that of the Zn^{2+} -treated enzyme, and that the addition of Ca^{2+} to the Zn^{2+} -treated enzyme helped make its active centre totally accessible to NADPH. These kinetic parameters are also in agreement with results showing that the Zn^{2+} effect does not seem to imply proteolysis. Although we have proposed that activation of the H_2O_2 generator by Ca^{2+} involves the release of an inhibitor [21], no such inhibitor has yet been found in the incubation medium after Zn^{2+} treatment of the particulate fraction. The present results indicate that Ca^{2+} may activate the H_2O_2 generator by a direct action on an auto-

Fig. 4. Effect of dithiothreitol on Ca^{2+} desensitization by ZnCl_2 treatment

Particulate fractions were first incubated with 0.5 mM- ZnCl_2 for 30 min at 40 °C. Controls were performed without dithiothreitol (■). Dithiothreitol (10 mM) was added at zero time (△) or after 20 min (▲). The reaction was stopped by adding 30 vol. of buffer A; the particulate material was collected by centrifugation, resuspended in buffer A and left for 24 h at 0 °C without (■) or with (△, ▲ and ●) 5 mM-dithiothreitol. The particulate material was collected and washed as described in the Materials and methods section and the H_2O_2 -generating activity assayed.

Fig. 5. Effect of Ca^{2+} on the kinetic constants of the Zn^{2+} -treated NADPH-dependent H_2O_2 generator (Lineweaver-Burk plots)

Particulate fractions were incubated with 0.5 mM- ZnCl_2 at 40 °C. The NADPH-dependent H_2O_2 activity was measured in the absence (○) and in the presence (●) of Ca^{2+} . The NADPH concentration was determined by measuring the absorbance at 340 nm.

inhibitory domain or an inhibitory protein component, thus making the active centre functional or accessible to NADPH.

We are grateful to Mrs. A. Lefèvre and Miss M. Sorgue for preparation of the manuscript.

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Received 31 July 1991; accepted 11 October 1991