Study on the generation mechanism of reactive oxygen species on calcium peroxide by chemiluminescence and UV-visible spectra

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ABSTRACT: In the present work, the generation mechanism of reactive oxygen species (ROS) on calcium peroxide (CaO2) was studied. A very intense chemiluminescence (CL) signal was observed when adding an aqueous solution of luminol or 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2α]-pyrazin-3-one hydrochloride (MCLA) to a suspension of CaO2. The ROS released on CaO2 were thought to be oxidizing agents leading to CL, and were characterized by CL, UV-visible (UV-vis) spectra and the effective scavengers of the special ROS. From experimental results, the hydroxyl (•OH) and superoxide (•O2−) radicals were suggested to exist on the surface of CaO2. A reaction scheme for the formation of the ROS on CaO2 was also proposed and discussed. Of more interest was the finding that the CaO2 which released the •OH and •O2− on the surface exhibited good transition properties compared with alkaline-earth metal peroxides of the same group (MgO2, BaO2).

INTRODUCTION

Calcium peroxide (CaO2) is a white or yellowish solid peroxide which has a high-energy peroxide covalent bond and can slowly decompose to release oxygen at a ‘controlled’ rate when in contact with hydrous media. CaO2 is one of the most temperature stable inorganic peroxides, which has the functions of bleaching, disinfecting and deodorizing besides its stable oxygen-releasing capability (1). Nowadays, as an environmentally friendly product, CaO2 is not only widely used in agricultural planting, aquaculture, foodstuff keeping and medical treatment, but also widely in sterilization, degradation and sewage treatment as an oxygen intensifier, bleach and antiseptic (2–5). The reaction mechanism of CaO2 is not clear, even though it is such a useful compound. In the last several years, many reports have offered different interpretations of the reaction mechanism of CaO2. Mura et al. (6) presumed that ROS were released on CaO2 and they also guessed the species of ROS as hydrogen peroxide (H2O2), superoxide anion (•O2−); Ramo et al. (7) explained that hydrogen peroxide anions (HOO−) were produced on CaO2, but did not carry out further investigations; Qin et al. (8) also presumed the existence of ROS on CaO2, and the production of ROS was also detected in cellular responses; Trokiner et al. and Pierlot et al. (9, 10) further treated CaO2 to obtain calcium peroxide diperoxohydrate (CaO2•2H2O2) and also proved, by monitoring the luminescence spectra, that CaO2•2H2O2 could release singlet oxygen (1O2) in the process. Obviously, the release of the ROS on CaO2 is the main factor that contributed to the effect of CaO2, but the details have not been clarified and needed further research.

Chemiluminescence (CL) is a powerful analytical technique that has excellent sensitivity, and a wide linear dynamic range and requires relatively simple and inexpensive instrumentation. Furthermore, CL is an effective method in research on ROS, because of the associated instantaneous nature of CL and the short lifetime of ROS radicals (11–16). Goto et al. (17) successfully investigated the CL mechanism of the alkaline-earth metal peroxides (MgO2, BaO2) with CL reagents (luminol and lucigenin), and demonstrated that the hydroxyl (•OH) and •O2− were released chiefly on the MgO2 and BaO2, respectively, with CL. According to our literature search, corresponding reports about CaO2 have not so far been made.
In the present study, the CL phenomenon of CaO$_2$ with chemiluminescence reagents was investigated with CL, UV-visible (UV-vis) spectra. Based on the results of CL and the effect of various free radical scavengers on the CL emission intensity, the possible mechanism of the generation of ROS on CaO$_2$ was investigated.

**MATERIALS AND METHODS**

**Reagents**

CaO$_2$ was obtained from Sigma Aldrich (St. Louis, USA). 3-Aminophthalhydrazide (luminol) was obtained from Alfa Aesar-A Johnson Matthey Company (Heysham, UK). 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydr0imidazo[1,2-α]pyrazin-3-one hydrochloride (MCLA) was bought from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). Nitro blue tetrazolium (NBT) was purchased from Nakalai Tesque Inc. (Kyoto, Japan). 1,4-Diazabicyclo[2, 2, 2]octane (DABCO) was obtained from Acros Organics (NJ, USA). Tryptophan was from Beijing Xinjingke Biotechnology Co. Ltd (Beijing, China). All the reagents used in these experiments were of analytical grade or higher and were used without further purification. Water was purified using a compact ultra-pure water system (18.3 MΩ/cm; Barnstead, IO, USA).

**Apparatus**

The batch method was carried out with an BPCL ultra-weak CL analyser (Institute of Biophysics, Chinese Academy of Science, Beijing, China) using a 3 mL glass cuvette. The signals were recorded by a computer equipped with a data-acquisition interface. Data acquisition and treatment were performed using BPCL software. Spectrophotometer studies were performed in 1 cm quartz optical cells using a UV-2401PC (Shimazu, Japan) spectrometer.

**Batch CL measurements**

CaO$_2$ (5 mg) was suspended in 400 μL H$_2$O or organic solvents at a specific concentration in a 3 mL glass cuvette, and then 10 μL of the specific-concentration CL reagents (luminol, 10$^{-7}$ mol/L; MCLA, 5 × 10$^{-5}$ mol/L) were injected into the system via a μL syringe through the upper injection port. Light-producing reactions were carried out in the cuvette and the CL profile and intensity were displayed and integrated for a 0.01 s interval.

**UV-vis absorption spectra measurements**

CaO$_2$ (5 mg) was added to the 8 mL plastic cuvette with 5 mL 10$^{-3}$ mol/L NBT aqueous solution or 5 mL 10$^{-4}$ mol/L cytochrome c, respectively. After several minutes, the NBT solution changed from yellow to blue; the blue precipitates in the cuvette were dissolved with anhydrous ethanol, and the organic phase was diluted to the required concentration, then detected using the UV-2401PC spectrometer having been transferred to a 1 cm quartz optical cell. The cytochrome c solution also changed from red to yellowish; some of the yellowish solution in the cuvette was diluted to the required concentration, then detected using the spectrometer, having been transferred to a 1 cm quartz optical cell.

**RESULTS AND DISCUSSION**

**Confirmation of ROS on CaO$_2$ by CL**

CL reactions based on the oxidation of luminescent reagents such as luminol, lucigenin and MCLA are the effective method for the detection of ROS (18). As is well known, luminol-amplified CL can offer high sensitivity and it has been widely used for the detection of rapid and complex responses of radicals in different systems. The possible mechanism of luminol–radicals CL is that radicals will react with the luminol to produce an excited aminophthalate, and then the aminophthalate decays to the ground state, emitting co-instantaneous CL when the ROS are released from the system (19–21). Actually, strong and rapid CL (about 3300 count) was emitted when we injected 10 μL 10$^{-7}$ mol/L luminol into 400 μL suspension of CaO$_2$ in water (Fig. 1). The strong CL phenomenon indicated that ROS was released on CaO$_2$. In order to acquire sufficient evidence, ascorbic acid, a well-known ROS scavenger (22–24), was chosen for further experiments. As shown in Fig. 1, considerable quenching of the CL signal was observed at a relatively low concentration (5 × 10$^{-7}$ mol/L) of ascorbic acid; moreover, quenching increased with its increasing concentration. The CL signal was completely restrained when
Generation mechanism of ROS on calcium peroxide

**Figure 3.** The CL intensity of MCLA on CaO2 and the effect of the scavengers for 1O2. Conditions: MCLA 5 × 10^{-5} mol/L; H2O 400 μL; DABCO 5 × 10^{-2} mol/L; tryptophan 5 × 10^{-2} mol/L.

...which eliminates carbon dioxide (CO2) to yield the excited species, and then the excited species decays to the ground state, emitting co-instantaneous CL (18). Actually, the CL phenomenon of MCLA on CaO2 was the same as that for luminol, and a rapid CL signal (about 600 count) was also emitted when we injected 10 μL 5 × 10^{-5} mol/L MCLA into the suspension of CaO2 in water (Fig. 3). The rapid CL signal not only further affirmed the existence of ROS, but also indicated that •O2− or 1O2, or even both, existed on the surface of CaO2.

Both DABCO and tryptophan, the special scavengers for 1O2 (28–30), were chosen for experiments in this system. The CL signals were not evidently changed, even when scavengers at a high concentration (0.05 mol/L DABCO or tryptophan) were added to the system of MCLA on CaO2 (Fig. 3). The results indicated that there is no 1O2 released on CaO2.

The reaction of •O2− with NBT, as the currently accepted method, has frequently been used for detecting •O2− (17). The •O2− released from various reactions can reduce the yellow dye (NBT) to its blue diformazan form, which is only slightly soluble in aqueous solution and quickly precipitates from reactions. The rate constant for the reduction of NBT by •O2− is about 5 × 10^4 L/mol/s (31, 32). This method was also chosen for these experiments. The colour change of the NBT solution in the cuvette from yellow to blue was very evident when CaO2 was added, and many blue precipitates were observed at the bottom after some time (Fig. 4A, B). The maximum wavelength (λmax) of the anhydrous ethanol solution of blue precipitates shifted to 550–600 nm after the colour of the NBT solution with CaO2 changed to blue, compared with the original NBT solution with yellow colour and without the λmax in UV-vis spectra (Fig. 5).
Figure 4. The colour changes of the NBT solution with CaO₂ (B) and without CaO₂ (A) and the cytochrome c with CaO₂ (D) and without CaO₂ (C). Conditions: NBT 10⁻³ mol/L; CaO₂ 5 mg; cytochrome c 10⁻⁴ mol/L.

Figure 5. The UV-vis absorption spectra of the NBT solution with CaO₂.

The above results of colour change of the NBT solution with CaO₂ and the λ_max shift of the blue precipitates indicated that NBT was reduced by the •O₂⁻ to its diformazan product, and also explained that the •O₂⁻ was released on CaO₂.

In fact, cytochrome c is also a special compound for detecting •O₂⁻ in a system without an effective oxidant; the •O₂⁻ released from the reactions can react with it and change the colour from red to colourless (17). The colour of cytochrome c in the cuvette was changed from red to yellowish when CaO₂ was added (Fig. 4C, D), but the λ_max of cytochrome c is not evidently shifted (Fig. 6) between the original solution and a solution with CaO₂.

As a result of the •O₂⁻, the colour became lighter (from red to yellowish), but the finding that the colour remained all the time and the λ_max shifted only slightly further validated the existence of the oxidative ROS. The result is consistent with the existence of •OH on CaO₂.

Figure 6. The UV-vis absorption spectra of the cytochrome c solution with CaO₂.

Possible CL mechanism of CaO₂

Based on the above experimental results and many previous studies, the possible generation mechanism of ROS on CaO₂ can be given as follows:

\[
\text{CaO}_2 + 2\text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \text{H}_2\text{O}_2 \quad (1)
\]

\[
\text{H}_2\text{O}_2 + e^- \rightarrow \cdot\text{OH} + \cdot\text{OH} \quad (2)
\]

\[
\cdot\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^- \quad (3)
\]

\[
\text{HO}_2^- \rightarrow \cdot\text{O}_2^- + \text{H}^+ \quad (4)
\]

As is well known, CaO₂ can decompose slowly in moist air [reaction (1)]. CaO₂ reacting with H₂O can slowly produce H₂O₂, so CaO₂ is considered to be ‘the solid peroxide hydrogen’. H₂O₂ can readily undergo redox by obtaining a single electron in the suspension of alkaline-earth metal peroxides to offer the •OH; reaction (2) has been proved by the addition of the special scavenger in previous investigations (17, 33). The succeeding reactions (3) and (4) can also occur in the system (34, 35). For the different alkaline-earth metal peroxides, the conditions of the suspension, such as solubility and pH value, are similar, so the possible reactive processes should also be similar. The primary difference in the generation of ROS on the different alkaline-earth metal peroxides rests with the reactive velocity of each step. For example, •OH was the main ROS on MgO₂, because the reactive velocity of reaction (3) was much slower than that of reaction (2); whereas •O₂⁻ was the main ROS on BaO₂, because the reactive velocity of reaction (3) was much faster than that of reaction (2) (17). The pH value for a 1% aqueous solution of CaO₂ was detected at about 12, so the HO₂⁻ transformed readily to the •O₂⁻ in strong basic conditions. Because of the short lifetime of ROS (e.g. •OH, ~10⁻⁶ s; •O₂⁻, ~0.1 s), they were quickly consumed by reactions (5) and (6) (34):
Table 1. ROS species on alkaline-earth metal peroxides

<table>
<thead>
<tr>
<th>Alkaline-earth metal peroxides</th>
<th>ROS species</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgO2</td>
<td>•OH</td>
</tr>
<tr>
<td>CaO2</td>
<td>•OH, •O2</td>
</tr>
<tr>
<td>BaO2</td>
<td>•O2</td>
</tr>
</tbody>
</table>

\[
\cdot \text{OH} + \text{HO}_2\cdot \rightarrow \text{H}_2\text{O} + \text{O}_2 \tag{5}
\]
\[
\cdot \text{O}_2^- \rightarrow \text{O}_2 + e^- \tag{6}
\]

From the above discussion, the sum reaction for CaO2 with water was proposed as reaction (7):

\[
\text{CaO}_2 + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2 + \text{1/2O}_2 \tag{7}
\]

In this study, not only •OH but also •O2 was confirmed on CaO2 by CL, UV-vis spectra and the effective scavengers of the special ROS. These interesting results indicated that both •OH and •O2 were the main ROS on CaO2. According to Table 1, CaO2, which released •OH and •O2, exhibited good transition properties compared with other compounds in the same group (IIA), i.e. MgO2 and BaO2, which only released •OH and •O2, respectively, on their surfaces. At the same time, this also indicated that the reactive velocity of reaction (3) was neither much slower (as MgO2) nor much faster (as BaO2) than that of reaction (2) for CaO2. The exhibited disciplinarian from MgO2 to CaO2 to BaO2 might rest with the metal and crystal lattice properties of the alkaline-earth metal peroxides as metal oxides (36), which needs further investigation in the future.

CONCLUSION

In this study, the ROS species and their generation mechanism on CaO2 were investigated by CL, UV-vis spectra and effective scavengers of the special ROS. From the experimental results, •OH and •O2 radicals were suggested to exist on the surface of CaO2. A reaction scheme for the formation of ROS on CaO2 was also proposed and discussed. Of more interest was the finding that CaO2, which released •OH and •O2 on the surface, exhibited good transition properties compared with alkaline-earth metal peroxides (MgO2, BaO2) of the same main group, which only released •OH and •O2, respectively, on their surfaces.

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