Production of OH-Radical-Type Oxidant by Lucigenin

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In the presence of NADH-reductases (dihydrolipoamide: NAD oxidoreductase E. C.1.8.1.4 from pig heart or from Clostridium kluyverii; frequently also addressed as "diaphorases") and NADH lucigenin strongly increases ethylene production from a-ketomethylthioibutyrate (KMB) as an indicator for strong oxidants of the OH-radical type. These reactions are further stimulated in the presence of Fe³⁺ ions. With these NADH-diaphorases, the structurally similar poison, paraquat, in the absence or presence of Fe³⁺ has no effect. With ferredoxin-NADH reductase (E. C.1.8.1.2.), however, paraquat reacts quasi identical to lucigenin. Superoxide dismutase, catalase, free radical- or OH-scavengers such as mannitol, propylgallate, DABCO, and desferal inhibit the reaction whereas EDTA (in the presence or absence of added Fe³⁺) is stimulatory. From these data we conclude that the superoxide – indicator LUC is redox-active after unspecific coupling to several almost ubiquitous NAD(P)H-reductases catalyzing monovalent oxygen reduction. Lucigenin thus should no longer be used as a "specific" superoxide indicator.

This report is in agreement with very recent results by Liochev and Fridovich (Arch. Biochem. Biophys. 337, 115 [1997]) and Vasquez-Vivar et al. (FEBS Lett. 403, 127 (1997)).

Introduction

Lucigenin (bis-N-methylacridinium) has been used as a “specific”, chemiluminescence-enhancing, superoxide indicator since many years by almost uncountable scientists in all fields of oxygen research. According to the medical literature-search system, “Medline Express”, by addressing the key words “lucigenin and superoxide”, ca. 250 reports are accounted in the past twenty years. Very recently two elegant studies by the Fridovich-Liochev (Liochev and Fridovich, 1997) and Kalyanaraman-Vasquez-Vivar et al., 1997) groups have documented that LUC itself produces superoxide. The Fridovich group used xanthine oxidase in the presence of NADH, lipoamide dehydrogenase or glucose oxidase as enzymic electron donors for LUC. With these systems stimulation of superoxide production as identified with spectrophotometric methods was shown. The Kalyanaraman group showed photometrically by NADPH consumption or with an ESR-spin trapping method that purified endothelial nitric oxide synthase (eNOS) produced a spin trap-superoxide adduct when eNOS was incubated in the presence of both LUC and NADPH. Both groups mention the structural similarity between PQ and LUC thus implicating similar reaction mechanisms.

It is therefore not too surprising that both compounds have been shown to induce enzymes in E. coli which are regulated by the soxRS regulon (Liochev and Fridovich, 1997a). Under certain conditions, superoxide can be converted into the extremely aggressive OH-radical. We showed (Youngman and Elstner, 1981) that PQ in the presence of ferredoxin-NADH reductase and NADPH catalyzed the production of an OH – type oxidant if either the oxygen tension was low or Fe-ions were present. This oxidant is also produced in the presence of ferredoxin or anthraquinone as autoxidizable electron acceptors (Elstner et al., 1980).

In this report we demonstrate that LUC couples with different NADH-dehydrogenases (E. C.1.8.1.4., often addressed as “diaphorases”) and ferredoxin-NADH reductase (E. C.1.18.1.2.) producing OH-radical- type oxidants which can be determined.

Abbreviations: CAT, catalase (E. C.1.11.1.6.); DABCO, diazabicyclo octane; DIA, diaphorase”, E. C.1.8.1.4. or E. C.1.18.1.2.; LUC, lucigenin; PG, propylgallate; SOD, superoxide dismutase (E. C.1.15.1.1.); PQ, paraquat.

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gaschromatographically by ethylene release from KMB (v.Kruedener et al., 1995).

Materials and Methods

Ethylene formation from KMB was followed as previously described (v. Kruedener et al., 1995).

A typical experiment was conducted as follows: 2 ml reaction volume contained: 1 ml 0.1 M phosphate buffer pH 7.2; 1.25 mM KMB; 75 µM NAD(P)H; Dia: 2.2 units (E. C.1.8.1.4.; pig heart; Boehringer) or 4 units (E. C.1.8.1.4.; C. kluveri; Serva) or 0.4 units ferredoxin-NAD reductase (E. C.1.18.1.2.; spinach, Sigma); Fe³⁺ (if indicated): 10 µM; PQ or LUC: 100 µM or as indicated; further additions as given in the individual figures. Ethylene was determined with the “head space technique” after 30 min reaction time as described earlier (v. Kruedener et al., 1995).

Enzymes or fine chemicals were from Boehringer (Mannheim), Serva (Heidelberg); Sigma (Deisenhofen) or Merck (Darmstadt).

All experiments have been repeated twice with n=4. Bars in Figures represent standard deviations.

Results

Ethylene formation from KMB in the presence of NADH and pig heart DIA is not influenced by PQ and stimulated by LUC. The reaction is enhanced in the presence of Fe³⁺-ions (Fig. 1). The basal rates of ethylene formation are between 600 and 1200 pmol/30 min in the system without electron acceptor due to slow autooxidation of the pig heart enzyme. These basal rates in the absence or presence of added Fe³⁺ are stimulated by increasing amounts of LUC up to 100 µM but not by PQ (Fig. 2 a,b).

In the absence of LUC as electron acceptor the presence of increasing amounts of NADH up to 75 µM increase the basal reaction; higher concentration of NADH are inhibitory (data not shown).

In the presence of LUC, however, maximal rates are obtained with 500 µM NADH independent of the presence or absence of Fe³⁺(data not shown).

Both in the presence of 100 µM LUC and in the presence or absence (data not shown) of Fe³⁺, SOD (E. C.1.15.1.1.), catalase (E. C.1.11.1.6.), desferal, mannitol, propylgallate, and DABCO strongly inhibit the reaction whereas EDTA is a stimulator doubling ethylene yield in the presence of Fe³⁺.

The best inhibitors are SOD and propylgallate yielding more than 90% inhibition (Fig. 3).

With C. kluveri- DIA almost identical results are obtained: LUC strongly stimulates ethylene release from KMB with maximal values at 250 µM LUC where PQ is without effect. Fe³⁺ stimulates the LUC reaction and has no effect on the PQ reaction (Fig. 4a, b).

Ethylene release from KMB catalyzed by ferredoxin-NAD reductase in the presence of NADPH is only slightly stimulated by LUC or PQ in the absence of Fe³⁺ but strongly in its presence. In this system, however, PQ is an effective electron acceptor yielding slightly higher ethylene production as compared to LUC Fig. 5).
Fig. 2. Concentration-dependent effects of paraquat or lucigenin on ethylene production from a-keto-methylthiobutyrate (KMB) in the presence of diaphorase (pig heart)
For reaction conditions see Fig. 1.
a) in the absence of Fe$^{3+}$
b) in the presence of 10 µM Fe$^{3+}$.

Fig. 3. Effects of various modulators of the “Haber-Weiss-chemistry” on lucigenin-stimulated ethylene production from a-keto-methylthiobutyrate (KMB) in the presence of diaphorase (pig heart).
For reaction conditions see Figure 2b; the LUC concentration was 100 µM, Fe$^{3+}$ was present at 10 µM.
Concentrations of the “modulators”: SOD, 100U; catalase, 100U; EDTA, 100 µM; desferal, 100 µM; DABCO, 10 mM; propylgallate, 1 mM; mannitol, 10 mM.
Discussion

LUC has been in use as a “sensitive and specific” detector of superoxide anion since approximately two decades in medical, animal and plant sciences. It exhibits the property to add $O_2^-$ after reductive conversion of the dication to the cation radical. The resulting dioxetane decays under light emission. It has been shown very recently and independently by two groups (Liochev and Fridovich, 1997; Vasquez-Vivar et al., 1997) that LUC similar to PQ autoxidizes after reduction producing superoxide. Therefore its utilization as a “sensitive and specific” detector for superoxide is no longer recommendable. Investigations on acti-
vated oxygen species potentially representing the toxic principle of the poison and herbicide, PQ, lead to the proposal (Elstner et al., 1980; Youngman and Elstner, 1981) of an electron-donor-\(H_2O_2\) complex, also addressed as “crypto OH”. This species is thought to represent an active “solvent cage” in analogy to the reactive species recently proposed for peroxynitrite, ONOOH, slowly decaying into \(NO_2\) and \(OH\) (Pryor and Squadrito, 1995). Paraquat in its monovalently reduced form has a high affinity for atmospheric oxygen and a redox potential of approximately +440mV. The estimated second order rate coefficient \(k_1\) for the reaction of the PQ cation radical \(\{PQ^+\}\) with molecular oxygen has been reported as \(8 \times 10^8 \text{ m}^{-3}\text{sec}^{-1}\) (Ledwith, 1977). The affinity of \(PQ^+\) for hydrogen peroxide is much lower as compared to molecular oxygen and the second order rate constant \(k_2\) of the reaction:

\[
PQ^+ + H_2O_2 \rightarrow k_2\text{ PQ}^2+ + OH^- + OH^-;
\]

is several orders of magnitude lower as compared to the one with oxygen (see above). The electron transfer towards \(H_2O_2\) yielding OH-radical is therefore close to nil in solutions well saturated with oxygen. We showed (Youngman and Elstner, 1981) that OH-radical-type oxidants produced at the expense of NADPH catalyzed by ferredoxin-NADP reductase and PQ is only measurable at low oxygen tensions or in the presence of added iron ions. In this report we compare the catalytic activities of PQ and LUC in cooperation with three different NAD(P)H-oxidoreductases: ferredoxin NADP reductase from spinach (E.C. 1.18.1.2.) and dihydrolipoamide: NAD oxidoreductase (“diaphorase”; E. C.1.8.1.4) from pig heart and from Clostridium kluyveri. As shown under results, both PQ and LUC catalyze ethylene release from KMB as an indicator reaction for the production of an OH-radical-type oxidant (v. Krüdener et al., 1995) if tested with ferredoxin-NADP reductase in the presence of NADPH. This reaction is strongly stimulated by the addition of \(Fe^{3+}\) (Fig. 5). This result is in agreement with our earlier report on PQ (Youngman and Elstner, 1981). In this system PQ and LUC behave almost identical. With both pig heart DIA or \(C.kluyveri\) DIA, PQ is inactive while LUC stimulates ethylene release from KMB in a reaction which is further stimulated by \(Fe^{3+}\)-ions (Figures 1, 2 and 4).

As shown in Figure 3 the reaction with pig heart DIA is strongly inhibited by SOD, catalase, desferal, propylgallate, DABCO and mannitol and stimulated by EDTA. This result is perfectly in line with the so-called “iron-catalyzed Haber-Weiss”- mechanism (see: Halliwell and Gutteridge, 1989). The finding that PQ in contrast to LUC is inactive with both the DIA-enzymes from pig heart and \(C.kluyveri\) cannot be due to different affinities towards molecular oxygen and hydrogen peroxide (see above) since with ferredoxin-NADP reductase both cofactors, PQ and LUC, are equally active. This difference therefore must be due to different affinities or redox potentials of the NADH-enzymes as compared to the NADPH-enzyme. Our results clearly support the suggestion made by Liochev and Fridovich (1997) and Vasquez-Vivar et al. (1997) that LUC should no longer be in use as a “sensitive and specific” detector of superoxide anion. In addition our test system allows a simple, rapid and relatively specific checking of earlier reports on “superoxide production” by biological systems in which the lucigenin method has been applied.


