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Research Article

On the Use of MCLA for Chemiluminescent Measurement of Reactive Oxygen Species

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ABSTRACT

Verification on the using of 2-methyl-6- (4-methoxyphenyl) -3,7-dihydroimidazo [1,2-a] pyrazin-3(7H)-one (MCLA) as a chemi-luminescence (CL) sensitizer for detection of reactive oxygen species (ROS) was done. In model experiments on aqueous solutions of MCLA (in the absence of cells), it was shown that its CL is quenched by a number of substances, normally contained in cells (iron ions, glutathione, ascorbate, oxygen, protons, etc.). It has been shown that CL of MCLA in water is enhanced by sodium azide, which is often used to block the mitochondrial respiratory chain. The data obtained should be taken into account when measuring of CL from MCLA or its analogs in cells will be done. A high sensitivity of CL of MCLA to oxygen, pH, ascorbate, glutathion and iron ions could allow use MCLA to detect these substances in solutions.

Keywords: Reactive oxygen species; Superoxide; Chemi- luminescence; (Methyl cypridina luciferin analogue); MCLA; Iron ions

INTRODUCTION

The most sensitive method to detect the reactive oxygen species (ROS) in a variety of systems, especially in biological ones, is chemi- luminescence (CL) [1]. Luminol was previously widely used to enhance the CL [2]. Later, other sensitizers, more effective, for example, 2-methyl-6- (4-methoxyphenyl) -3,7-dihydroimidazo [1,2-a] pyrazin-3 (7H) -one, also called methyl Cypridina Luciferin analogue (MCLA), were applied.

It has become generally accepted that MCLA is a specific probe for ROS [3-7]. Of course, MCLA reacts well with ROS, especially with superoxide. But as far as specificity is concerned, this is highly doubtful, since no evidences have been provided in this regard in the first papers [6,7]. It is not accident that in the article [8] the specificity of MCLA was questioned.

Earlier we showed [9] that another sensitizer-DCF-DA (2',7'-dichlorofluorescein diacetate), widely used for ROS measurement, and its deacylated product in cells change their

fluorescence properties under the influence of NADH and ascorbic acid (apparently, NADH and ascorbate simply suppress the conversion of DCF-DA to CM- H2DCFDA).

The purpose of this work is to test the specificity of MCLA, namely the establishment in the model experiments of the influence (or lack of influence) of a number of biologically important substances on CL of MCLA in aqueous solution (without the cells).

MATERIALS AND METHODS

The measurements were carried out on the Lum-5773 chemi-luminometer (MSU, RF) in the photon counting mode. We used freshly prepared aqueous solutions of MCLA (Sigma) at a concentration of 1 M at pH 5.5. MCLA was dissolved in DMSO and then added (in small quantity) to water to obtain the 1 M. The addition of substances (iron ions, glutathione, ascorbate, etc.) was carried out directly into this MCLA solution with stirring, and the kinetics of CL was immediately measured at 21°C.

RESULTS AND DISCUSSION

A possible mechanism for the CL of MCLA was described [10]. The reaction of chemo-sensitization takes place in the presence of molecular oxygen (Figure 1) [10]. It follows from this scheme that MCLA and its analogues glow themselves. In fact, these are

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unstable substances that react quickly not only with superoxide, but also with the molecular oxygen (presented in a solution). As a result unstable peroxide intermediate is formed and decays with the release of a quantum of light.

It immediately follows that the intensity of CL of MCLA in the cells will be proportional to the concentration of oxygen, but not only to the number of active forms of oxygen. In different intracellular organelles the oxygen concentration is different. In addition, concentration of the oxygen is decreased dramatically under breathing of mitochondria. Certainly, CL of MCLA will react on this.

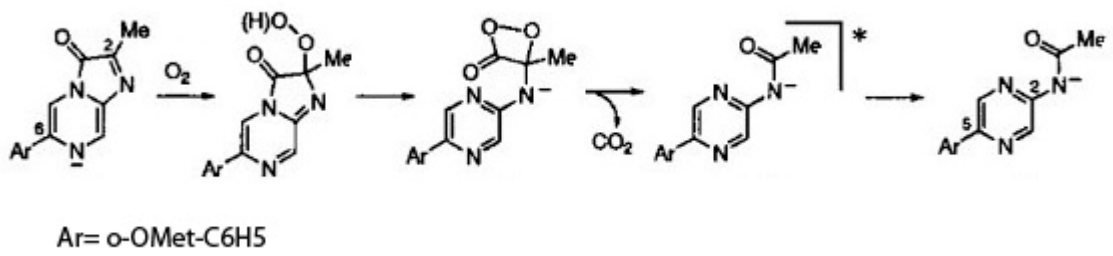

Figure 1: Possible mechanism of occurrence of CL of MCLA in the presence of oxygen.

We observed that when MCLA (dissolved initially in DMSO and added in a small amount to water) falls into an aqueous solution, an autoxidation reaction takes place accompanied by a sharp flash, and, after a few minutes, the CL goes to a plateau (data not shown). Authors Kambayashi et al. [8] also noticed this effect and they pre-incubated MCLA before addition to cells.

The protonated form of MCLA loses the ability to radiate [10]. This means that MCLA is highly sensitive to pH. Indeed, in our control experiments with added alkali or acid, it is clearly seen (Figures 2A and 2B) that CL of MCLA strongly depends on pH. With the addition of a small amount of sodium hydroxide, the intensity of CL is increased, since the proton block is removed (Figure 2A and Table 1). When adding hydrochloric acid, the intensity of CL, on the contrary, is decreased.

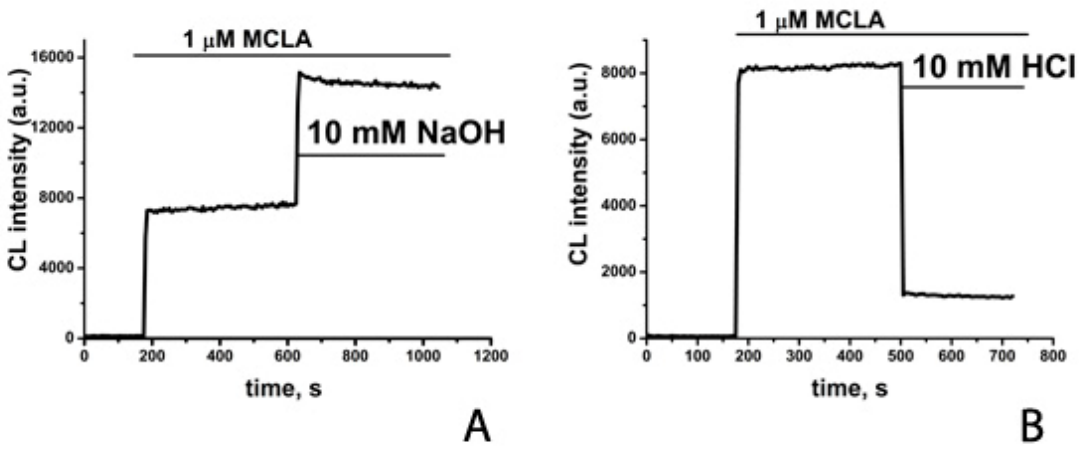

Figure 2: A) Effect of 10 mM NaOH on the intensity of CL of MCLA in aqueous solution. B) Effect of 10 mM HCl on the intensity of CL of MCLA in aqueous solution.

In protonated form, MCLA does not react with oxygen and is therefore more stable (it is no accident that MCLA powder is produced and stored in the composition with hydrochloride, Figure 2B). It follows that any change in the intracellular pH in the cellular structures will lead to a change in the CL of MCLA.

Strong reducing agents-ascorbic acid and reduced glutathione-markedly reduce CL of MCLA. Glutathione strongly quenches the CL of MCLA at a concentration of only It immediately follows that the intensity of CL of MCLA in the cells will be proportional to thconcentration of oxygen, but not only to the number of active forms of oxygen. In different intracellular organelles the oxygen concentration is different. In addition, concentration of the oxygen is decreased dramatically under breathing of mitochondria. Certainly, CL of MCLA will react on this.

Less powerful reductants, NADH and succinate, do not affect CL of MCLA (Table 1). When MCLA was added to 96% isopropyl alcohol, its CL intensity was 7.5 times higher

compared to the addition to water (data not shown). Perhaps this is due to much higher oxygen content in alcohol than in water. On the contrary, quenching of CL of MCLA occurs after addition of isopropanol in a small amount (1-3%) to water (Table 1 and Figure 3A). This means that the presence of any alcohols in cells, even in small amounts, will significantly affect the CL of MCLA.

Table 1: The effect of various substances on the intensity of CL of MCLA in aqueous solution. The concentration of MCLA was 1 μM, pH 5.5.

S.no	Substance	Concentration, mM	Chemi-luminescence of MCLA, %
1	MCLA, water	0.001	100
2	NaOH	10	198
3	HCl	10	16.5
4	Ascorbic acid	0.01	41
5	Glutathione reduced	0.01	38.4
6	Glutathione reduced	0.05	12.5
7	NADH	0.3	100
8	Succinate	5	100
9	Sodium azide	5	184
10	FeSO4 (II)	0.001	89.3
11	FeSO4 (II)	0.01	55.8
12	FeSO4 (II)	0.1	11.3
13	Isopropanol	3%	37.6

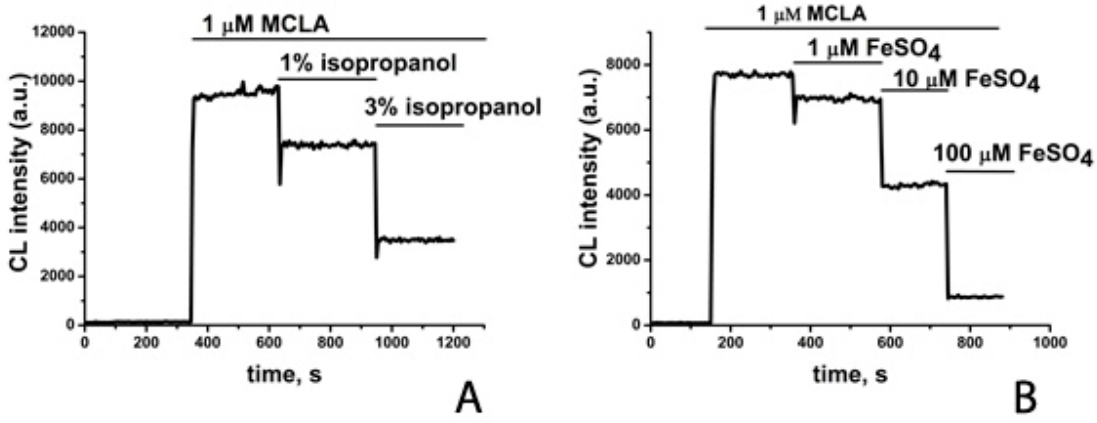

Figure 3: A) Influence of different concentrations of isopropanol on the intensity of CL in MCLA in aqueous solution. B) The effect of various concentrations of $FeSO_4$ on the intensity of CL in MCLA in aqueous solution.

We found a significDnt increase in CL of MCLA in water by the addition of 5 mM sodium azide (Table 1). Azide is often used in the analysis of the functioning of respiratory chain of mitochondria for blocking of cytochrome C oxidase. Interestingly, a decrease in CL of MCLA conversely was noted in the presence of azide in studies on cells [11,12].

CL of MCLA is strongly quenched by the iron ions (Figure 3B and Table 1), since iron is a paramagnetic substance. Its effect

on the CL of MCLA begins already with 1 μM at an equimolar amount. His automatically means that iron ions form with MCLA a stable complex, within which occurs deactivation of excitation. The intensity of CL of MCLA was strongly dependent on the concentration of iron in a very wide range. This means that, in principle, it is possible to make a test system with MCLA for measuring the concentration of iron ions in solutions.

All of the above study does not mean that you should generally refuse MCLA. It can be used to detect glutathione and iron ions in solutions, without cells. In the case of cells, it is important for researchers to consider the possibility of direct influence (activation or vice versa deactivation) on CL of MCLA by these biological active substances.

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