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Mechanistic aspects of detection of reactive oxygen and nitrogen species with the use of selected fluorogenic probes

Direct detection of reactive oxygen and nitrogen species (ROS/RNS) can be difficult in biological samples because of a short lifetime of these species related to their high reactivity. Nevertheless, in simple and strictly controlled *in vitro* environment, direct detection of ROS/RNS is possible with the use of fast time-resolved methods, *e.g.* pulse radiolysis and flash photolysis. On the other hand, when one wants to detect reactive oxygen and nitrogen species using cell cultures or *in vivo*, the use of indirect methods based on specially designed spectroscopic probes is highly recommended. Among different approaches, fluorogenic probes have been widely used for ROS/RNS measurements, mostly due to the variety of probes and the versatility and sensitivity of fluorescence-based techniques. These probes react with the reactive oxygen and nitrogen species of interest to give fluorescent products. The ideal fluorescent probe is selective towards detected species, reacts directly with it, and gives an easy to detect and stable product of the reaction. However, despite the growing power of ROS and RNS detection the interpretation of experimental data is very often erroneous due to the lack of understanding of the chemistry behind the assay. Thus, not only the selectivity and specificity of the probe toward particular free radical species should be known, but also the reaction mechanism, the redox chemistry of generated species, and the effect of other components of the cellular system must be evaluated and understood.

In PhD thesis titled "Mechanistic aspects of detection of reactive oxygen and nitrogen species with the use of fluorogenic probes" the reactivity of fluorogenic probes dedicated for the detection of superoxide radical anion ($O_2^{\bullet -}$) and nitrogen oxide ($^{\bullet}NO$) is described. It is widely believed that, detection of those species with fluorogenic probes such as

hydroethidine, requires "the activation step" *via* one-electron oxidation reaction, which makes this step crucial for whole reactivity of these probes towards superoxide and nitric oxide.

In the first part of this work, a complex spectroscopic characterization of one-electron oxidation products of HE and its analogs, benzidine (Benz), methylethidine (MeE) and N,N,N',N'-tetramethyl-hydroethidine (TMeHE), generated by pulse radiolysis in low-temperature ionic liquid matrices, is presented. Pulse radiolysis experiments showed, that the main one-electron oxidation product of HE and its analogs is radical cation. These results are supplemented by quantum mechanical calculations with time dependent density function theory (TD-DFT). Calculated electronic spectra of radical cations and other possible one-electron oxidation products of HE and its analogs were in good agreement with that obtained from pulse radiolysis experiments, what further confirmed the identity of observed species. Quantum mechanical calculations results showed, that the highest spin density is localized at carbon atom C-2 of the phenanthridine ring, consistent with the position of hydroxylation of the probe by superoxide. This observation lead to the conclusion, that the affinity of nucleophilic $O_2^{\bullet-}$ to the radical cations of HE and its analogues should strongly depend on the spin density at position C-2, and steric hindrance around the site of nucleophilic attack, which should be taken into account during development of new analogs of the HE probe.

In the second part of this work, the reactivity of HE towards biologically important one-electron oxidants is described. Presented rate constants of reactions of HE with biologically relevant oxidants, e.g., glutathionyl radicals ($GS^{\bullet}$), $^{\bullet}NO_2$, and $CO_3^{\bullet-}$ were determined by pulse radiolysis. Because of fast dismutation reaction of superoxide radical anion direct determination of its reaction rate with HE is rather problematic and erroneous. In this work, alternative method of determination of this reaction rate constant, from the dependence of rate constant of HE reaction with selected chloromethylperoxyl radicals ($CH_2ClO_2^{\bullet}$, $CHCl_2O_2^{\bullet}$, $CCl_3O_2^{\bullet}$) on their standard reduction potential, is presented.

Additional liquid chromatography experiments, on the oxidative conversion of HE to 2-OH-E^+, are described. It was shown that the formation of the fluorescent product depends strongly on the yield of one-electron oxidation of HE to its radical cation. In short conclusion, this unspecific one-electron step can lead to the sensitization of the probe to superoxide radical

anion. The influence of horseradish peroxidase, superoxide dismutase, glutathione and ferric ions on the yield of 2-OH-E^+ formation is described.

In the last chapter of this work the mechanistic aspects of nitrosation of diamine probes are described. It was shown that superoxide radical anion affects the detection of $^{\bullet}NO$ with diamine probes. It was also shown that the nitrosation yield of 2,3-diaminonaphatalene and spirolactam rhodamine B derivative (RhPDA) depends on $O_2^{\bullet-}/^{\bullet}NO$ ratio. The influence of low molecular compounds, *e.g.* glutathione, azide and carbonate anions on the yield of nitrosation was also investigated.

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