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MONITORING OF ENZYMATIC ACTIVITY IN SITU BY EPR

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A novel approach using an EPR method for the determination of activity of enzymes, whose substrates or products are low molecular weight compounds containing SH-groups, is proposed. The approach is based on thiol-disulfide exchange of stable nitroxyl biradical containing a sulfur-sulfur bond with low molecular weight thiol. The method is applied to determine activity of acetylcholinesterase in a larvae bollworm (from the formation of thiocholine from acetylthiocholine). The method is characterized by high sensitivity (up to 2×10^{-12}m thiocholine) and makes possible measurements in optically unclear (scattering and coloured) media and determination of enzymatic activity (ca. 1 min) directly in the homogenate obtained from the heads of individual larvae. Thus, the method can be recommended for the fast monitoring of many enzymatic systems (including glutathione-dependent) directly in biological tissues of warm-blooded animals and insects.

The activity of a series of enzymes can be determined by examining the concentration change of SH-containing substrate or product during the enzymatic reaction. Of these series of enzymes, glutathione-dependent enzymes and acetylcholinesterase (AChE, EC 3.1.1.7) should be mentioned in the first place. AChE is one of most important enzymes which is responsible for the transfer of nervous impulses in insects and warm-blooded animals [1]. AChE is an important subject for toxicological studies since it is the target of action of organophosphate and carbamate pesticides. The most widely used method of AChE activity determination involves measuring the content of thiocholine (TCh) accumulated during the course of enzymatic hydrolysis of acetylthiocholine (ATCh). TCh content is determined spectrophotometrically using 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Ellman reagent) [2]. As is known,

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Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine; TCh, thiocholine; CA, cysteamine.

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measurements with DTNB can be made only in optically clear solutions, the sensitivity of the method being insufficient for many cases.

Recently we have proposed a novel method for the quantitative determination of SH-containing compounds, based on thiol-disulfide exchange of stable nitroxyl biradical ṘS–SṘ with thiols [3]. This method has been used to determine concentrations of cysteine and glutathione in mouse and rat blood [3]. In this paper the biradical ṘS–SṘ has been first applied for determining enzymatic activity. The use of EPR as a detecting technique permitted us to measure the ATCh hydrolysis rate during the enzymatic reaction directly in the homogenate obtained from the head of an individual larvae and to recommend this method for fast monitoring of enzymatic activity directly in cells, tissues, etc.

EXPERIMENTAL

Materials: Biradical ṘS–SṘ was synthesized as described in [3]. ATCh chloride; DTNB; diisopropyl fluorophosphate, DFP; phenylmethylsulfonyl fluoride, PMSF; sodium dodecylsulphate, SDS (Sigma) and CA cysteamine hydrochloride (Fluka) were used without additional purification.

Fifth instar bollworm *Heliothis armigera* Hbn (Laboratory Population, Institut of Chemical Kinetics and Combustion, Novosibirsk) were used. The homogenate was obtained from the head of individual insect in 10 mM Na-phosphate buffer, pH = 7.5 in a glass homogenizer (200 μl buffer per 1 head).

Optical measurements: The protein concentration in the homogenate was determined according to Lowry et al. [4]. AChE activity was measured according to Ellman et al. [2]. The reaction was carried out at 23°C for 30 min and stopped by adding 1% SDS. Prior to absorption measurements at 412 nm ($\varepsilon = 1.4 \times 10^4$ M^{-1} cm^{-1} [5]), the samples were centrifuged for 4 min at 15000g. All optical measurements were performed on Ultraspec II Spectrophotometer (LKB).

EPR measurements: To determine the kinetics of the reaction of ṘS–SṘ with CA, the biradical solution in buffer of a given pH was mixed with an equal volume of CA solutions in the same buffer in a mixer placed inside an EPR spectrometer resonator (mixing time ~ 1s).

To determine AChE activity by EPR, the reaction mixture was prepared by successive additions of the necessary quantities of the homogenate and ATCh in 10 mM Na-phosphate buffer, pH = 7.5 to 10 μl of the biradical ṘS–SṘ solution in dimethylsulfoxide, DMSO, so that the final volume was 200 μl. It was found that the addition of up 5% DMSO to the reaction mixture had no effect on the AChE activity.

In the inhibition assay for AChE activity the inhibitors PMSF and DFP dissolved in DMSO were added and preincubated prior (10 min) of the enzymatic reaction.

The EPR spectra were recorded at room temperature on an ER-200 D-SRC (Bruker) or E-12 (Varian) spectrometers in a flat cell or in a mixer of 200 μl volume.

RESULTS AND DISCUSSION

The structure of stable nitroxyl biradical used in the study is shown in Fig. 1. The presence of the disulfide bond in the biradical allows this compound to participate in thiol-disulfide exchange reaction with SH-groups of low- and high-molecular weight compounds (B-SH).

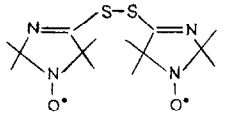

Fig. 1. Structure of biradical ṘS–SṘ. Synthesis and properties of the biradical are given in ref. 3.

$$\dot{R}S - S\dot{R} + B\text{–}SH \underset{K_r}{\overset{K_f}{\rightleftarrows}} \dot{R}\text{–}SH + BS\text{–}S\dot{R} \qquad (1)$$

Figure 2 shows the EPR spectrum of the biradical ṘS–SṘ in which the effect of the interaction of spins of two nitroxyl fragments can be seen (components 4,5,6,7,8,9). Note, that the components 1,2 and 3 coincide with those of the corresponding monoradical. When CA which is assumed to be a thiocholine analogue, is added to the biradical solution, the EPR spectrum of ṘS–SṘ transforms to the unperturbed monoradical nitroxyl EPR spectrum (Fig.

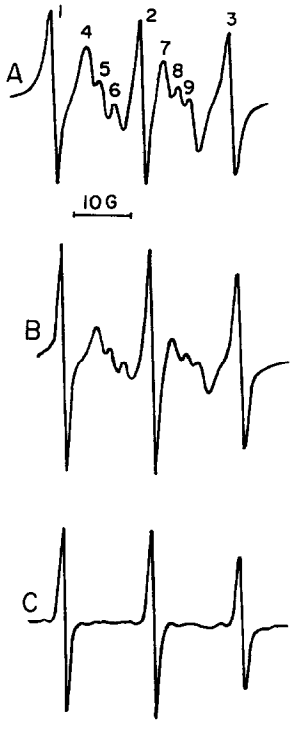

Fig. 2. The EPR spectra of 100 μM ṘS–SṘ solution in 10 mM Na-phosphate buffer, pH = 7.5. Experimental conditions were the same except for the gain. Microwave power -20 mW, modulation amplitude -1G. (A) - The initial EPR spectrum, gain 5×10^4. (B) - The spectrum after addition of 1.5×10^{-5}M cysteamine, gain 2.5×10^4. (C) - The spectrum after addition of 10^{-3}M cysteamine, gain -3.2×10^3.

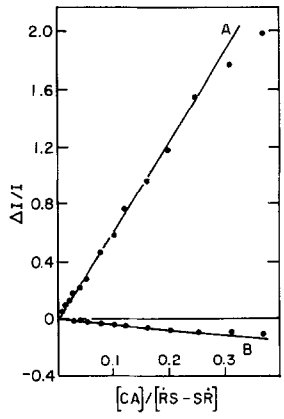

Fig. 3. The dependence of the relative increase of peak intensity component 1 $I\text{-}I_1/I_1$ (A) and decrease of peak intensity component 4 $I\text{-}I_4/I_4$ (B) of the biradical EPR spectrum (see Fig. 2) on the cysteamine/biradical ratio. Biradical concentration is 100 μM in 10 mM Na-phosphate buffer, pH = 7.5.

2), i.e., reaction (1) takes place. In this instance, while biradical components 4,5,6,7,8,9 tend to decrease, monoradical components 1,2,3 increase. We have shown before [3] that the integrated intensities of all components of the biradical EPR spectrum remain unchanged, in other words, at the reagents concentrations employed the reduction of ṘS – SṘ with CA SH-groups does not occur. Reaction (1) is reversible. We have studied in detail the interaction of the ṘS – SṘ with CA. Fig. 3 shows the dependence of the relative decrease of the biradical component ($\Delta I_4/I_4$) and corresponding increase of monoradical one ($\Delta I_1/I_1$) on [CA]/[ṘS–SṘ] ratio. It is seen that up to the ratio [CA]/[ṘS–SṘ]$\lesssim$ 0.3 these dependences are linear and thus the reverse reaction in (1) can be neglected. The data in Figs. 2 and 3 show an increase in peak intensity of the resulting monoradical component to be ~16 times as high as that of the biradical decrease. Naturally, the integrated intensity is preserved. Therefore it is convenient to monitor reaction (1) by the accumulation of monoradicals.

The k_f value at various pHs have been calculated by analyzing the kinetics of reaction (1) of CA with the biradical (Table 1). A strong dependence of k_f on pH testifies to the fact that the basic mechanism of reaction (1) is the interaction of ṘS–SṘ with the CA as mercaptidione. Note that the interaction of ṘS–SṘ with cysteine and glutathione yields similar k_f values [3].

Consider the main requirements of the detection of enzymatic activity by the aid of biradical ṘS–SṘ using AChE as an example. In the presence of the biradical the enzymatic process is followed by the splitting of the disulfide bond ṘS–SṘ:

Table 1. Bimolecular rate constant k_f for the thiol-disulfide exchange reaction (1) of biradical ṘS–SṘ with cysteamine at various values of pH

pH	4.0	4.67	5.34	7.5
k_f (M^{-1} s^{-1})	61 ± 6	92 ± 10	290 ± 30	$\gtrsim 10^4$

The kinetics of reaction (1) were determined from the increase of peak intensity of component 1 of the biradical EPR spectra. The k_f value was determined by analyzing the initial part of the kinetic dependence. The final concentrations of reagents were [ṘS–SṘ]=2.5×10^{-5}M, [CA]=(0.5-1) × 10^{-6}M.

$$ATCh + AChE \xrightarrow{K_m, V_{max}} TCh + AChE + CH_3COOH \quad (2)$$

$$TCh + \dot{R}S{-}S\dot{R} \underset{K_r}{\overset{K_f}{\rightleftarrows}} TCh - S\dot{R} + \dot{R}{-}SH \quad (3)$$

The rate of disulfide splitting via reaction (3) is certain to reflect the rate of enzymatic process (2) provided two requirements are fulfilled: first, the reaction mixture must contain an excess of biradical so that the reverse reaction in (3) can be neglected; second, the characteristic time of TCh disappearance according to reaction (3) must be substantially faster, than that of the TCh formation in reaction (2). Since enzymatic reaction (2) has been studied at pH 7.5, the following estimate of the characteristic time of the disappearance of CA and its analog TCh according to reaction (3) may readily be obtained: $\tau \lesssim 1/k_f \cdot [\dot{R}S{-}S\dot{R}] \simeq 1s$ (at $k_f \simeq 10^4 M^{-1} s^{-1}$ and $[\dot{R}S{-}S\dot{R}] = 10^{-4}M$).

Thus the biradical ṘS–SṘ can be used to measure the enzymatic reaction (2) rate if the amount of the resulting product TCh is no more than 0.3 of the biradical amount (Fig. 3) and the characteristic time of TCh product formation is considerably greater than 1s. These conditions are easy to fulfill and maintain under experimental control.

At neutral pH 7.5, i.e., under conditions of enzymatic reactions, ṘS–SṘ decomposes spontaneously to monoradical derivatives. This reaction rate is, however, low (Fig. 4, curve 1), $\tau_{1/2} \approx$ 18 hrs. Addition of the homogenate, obtained from the head of individual larvae, to the reaction mixture causes the formation of monoradical, probably, due to reaction of biradical with protein SH-groups (Fig. 4, curve 2). A faster initial part of the curve may be attributed

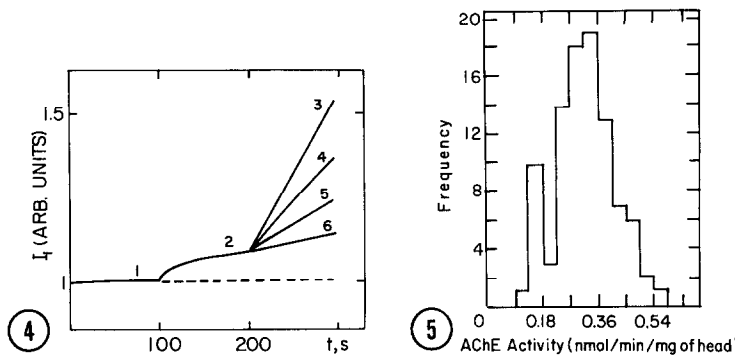

Fig. 4. The time-course of the peak-intensity of component I of the biradical EPR spectrum (see Fig. 2). ṘS–SṘ concentration - 100 μM in 10 mM Na-phosphate buffer, pH = 7.5.
1) The initial ṘS–SṘ solution; 2) after addition of homogenate, obtained from the head of larvae, protein concentration - 0.15 mg/ml; 3) after addition of ATCh - 4×10^{-4}M; 4) before adding of the ATCh the inhibitor, DFP, was added. DFP=3.4×10^{-4}M (4), DFP–6.6×10^{-4}M (5), DFP–2×10^{-3}M (6).

Fig. 5. The frequency distribution of individual AChE activity. The rate of TCh formation by the homogenate, obtained from the head of individual larvae, was measured. In experiments 69 individual insects were used.

to the interaction with the most accessible surface protein SH groups. In the presence of ATCh (Fig. 4, curve 3), the formation of monoradicals is accelerated by the enzymatic hydrolysis of substrate, the kinetic process is linear and AChE activity is easily determined. Note that a possible modification of the AChE sulfhydryl groups by the biradical has no effect on its activity. This has been experimentally verified by comparing the TCh amount formed via the enzymatic reaction in the presence of the biradical and in its absence, when ṘS–SṘ was added after ATCh just before measurement. This observation is in agreement with recently published data on a slow deactivation of AChE from *Torpedo Californica* due to the action of the reagents on its SH groups [6].

The observed AChE activity is inhibited by the known inhibitors of cholinesterase [7,8] DFP (See Fig. 4) and PMSF (data not shown). For DFP $IC_{50} = 4\times10^{-4}$M, for PMSF $IC_{50} = 3.3\times10^{-4}$M.

The method proposed was applied to determine kinetic characteristics of acetylcholinesterase activity from the heads of larvae bolloworm: $K_m = (30\pm5)$ μM; $V_{max} = (12.3\pm2)$ nmol/min per mg of protein. These results are in good agreement with ours obtained by Ellman reagent (for the same preparation): $K_m = (23\pm5)$ μM; $V_{max} = (10.5\pm2)$ nmol/min per mg of protein. These values of kinetic parameters are characteristic of enzymatic hydrolysis of ATCh by acetylcholinesterases from different sources [1,9].

Thus, the EPR method allows the enzymatic activity determination of a very small AChE quantity in optically unclear and colored solutions. Due to the EPR method the quantity of TCh formed by hydrolysis of 4×10^{-4}M ATCh can be measured for 1 min at $\sim10^{-3}$ mg/ml of protein in homogenate (to be compared with ~1 mg/ml of protein in Ellman's method). This permits the acetylcholinesterase activity in individual insects to be found without preliminary removal of large particles from the homogenate. Experiments were performed on the homogenates obtained from the heads of larvae bollworm (Fig. 5). The average initial rate of ATCh hydrolysis per head weight was 0.33 ± 0.1 nmol/min per mg of head. At the same time the distribution of individual AChE activities shown in Fig. 5 may indicate two groups of insects with a range of AChE activities.

The proposed method makes it possible to monitor reliably the formation of thiocholine beginning from ca. 10^{-8}M concentrations using a routine EPR-spectrometer. Taking into account the volume necessary for EPR measurements (100-200 μl), the absolute sensitivity of the method is $\lesssim 2\times10^{-12}$m of TCh.

It is known that the use of stable nitroxyl radicals in real biological systems (cells, tissues, etc) is often complicated by radicals reduction with donors of electrons to diamagnetic hydroxylamines (see for example, ref. 10). We did not observe reduction of biradicals $\dot{R}S-S\dot{R}$ and monoradicals during AChE activity measurements (control with $K_3Fe(CN)_6$). This observation can be explained by (1) the high reaction rate of thiol-disulfide exchange at pH = 7.5 (see Table 1) and (2) small amounts of the biological materials used in experiments.

We hope that the proposed method will allow one to determine not only absolute concentrations of glutathione in cells, but also rates of many important enzymatic reactions (e.g. glutathione-dependent ones) directly in biological samples: cells, tissues, etc.

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