Chemico-Biological Interactions 203 (2013) 63–66

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

The specific interaction of the photosensitizer methylene blue with acetylcholinesterase provides a model system for studying the molecular consequences of photodynamic therapy

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ARTICLE INFO

Article history:
Available online 15 November 2012

Keywords:
Acetylcholinesterase
Methylene blue
Photosensitizer
Photodynamic therapy
Mass spectroscopy
Singlet oxygen

ABSTRACT

The photosensitizer, methylene blue (MB), generates singlet oxygen ($^1O_2$) that irreversibly inhibits *Torpedo californica* acetylcholinesterase (*Tc*AChE). In the dark MB inhibits reversibly, binding being accompanied by a bathochromic shift that can be used to show its displacement by other reversible inhibitors binding to the catalytic 'anionic' subsite (CAS), the peripheral 'anionic' subsite (PAS), or bridging them. Data concerning both reversible and irreversible inhibition are here reviewed. MB protects *Tc*AChE from thermal denaturation, and differential scanning calorimetry reveals a ~8 °C increase in the denaturation temperature. The crystal structure of the MB/*Tc*AChE complex reveals a single MB stacked against W279 in the PAS, pointing down the gorge towards the CAS. The intrinsic fluorescence of the irreversibly inhibited enzyme displays new emission bands that can be ascribed to *N'*-formylkynurenine (NFK); this was indeed confirmed using anti-NFK antibodies. Mass spectroscopy revealed that two Trp residues, Trp84 in the CAS, and Trp279 in the PAS, were the only Trp residues, out of a total of 14, significantly modified by photo-oxidation, both being converted to NFK. In the presence of competitive inhibitors that displace MB from the gorge, their modification is completely prevented. Thus, photo-oxidative damage caused by MB involves targeted release of $^1O_2$ by the bound photosensitizer within the aqueous milieu of the active-site gorge.

1. Background

Photodynamic therapy (PDT) is being increasingly adopted as a technique for selective local destruction of malignant tumours [1,2]. This treatment involves administration of a photosensitizer, which, upon illumination, generates singlet oxygen ($^1O_2$) that damages biomolecules in its close vicinity, including lipids, proteins and nucleic acids [3]. The photosensitizers currently used in PDT are mostly porphyrins, but they lack specificity for the target cells, and are activated at short wavelengths that generate non-specific damage. These shortcomings can be overcome by using targeted photosensitizers that are excited at longer wavelengths. However, their optimal utilization will require knowledge of their specificity. This, in turn, will necessitate the development of satisfactory model systems.

Methylene blue (MB) (Scheme 1) is a classical photosensitizer that has been utilized for the treatment of a broad spectrum of diseases [4]. It was shown already by Augustinsson that MB serves as a powerful reversible inhibitor of acetylcholinesterase (AChE) in the dark [5], and this interaction was more recently further characterized for both AChE and butyrylcholinesterase (BChE) [6]. Kochevar and coworkers showed that the photosensitizer rose bengal irreversibly inhibited human erythrocyte AChE under illumination [7]. Data concerning both reversible and irreversible inhibition are reviewed below.

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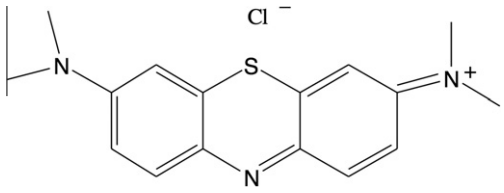
http://dx.doi.org/10.1016/j.cbi.2012.10.021



Scheme 1.

In recent years we have utilized *Torpedo californica* AChE (*Tc*AChE) as a model system for studying various types of radiative damage [8–10]. It occurred to us that the tight interaction of MB with *Tc*AChE could provide an ideal experimental system for studying the local oxidative damage produced by $^1O_2$ generated *in situ*, within the active site of a protein.

2. Results

2.1. Reversible inhibition of TcAChE by MB and structural characterization of the MB/TcAChE

As mentioned above, MB is known to serve as a reversible inhibitor of AChE. This was confirmed for *Tc*AChE, using the Ellman assay, with acetylthiocholine as substrate [11]. MB was indeed shown to act as a non-competitive inhibitor of the *Torpedo* enzyme, with K_i = 33 nM [12].

In view of the fact that non-specific photoactive damage is wavelength-dependent, we decided to examine the effect of *Tc*AChE on the spectral characteristics of MB. MB itself displays an absorption maximum at 662 nm, and undergoes a bathochromic shift, to 682 nm, upon interaction with the enzyme, with an isosbestic point at 671 nm. Fig. 1 shows an experiment in which MB was titrated with increasing levels of *Tc*AChE until saturation had been achieved. The pronounced bathochromic shift permitted us to monitor the displacement of bound MB by a series of competitive inhibitors for which both the kinetic characteristics and X-ray structural data for their complexes with the enzyme were known [12]. Such data were obtained, in particular, for an inhibitor specific for the 'anionic' subsite of the catalytic site (CAS) at the bottom of the active-site gorge, edrophonium (EDR), for one specific for the peripheral 'anionic' site (PAS) at the entrance to the gorge, propidium (PROP), and for the gorge-spanning bifunctional ligand, BW284c51 (BW), that bridges the CAS and the PAS [13,14]. Both the CAS- and PAS-directed inhibitors could displace the MB from the gorge, but full displacement could only be achieved by using the two together, or by an excess of BW.

In general, reversible inhibitors stabilize *Tc*AChE against thermal denaturation [15]. Fig. 2A shows the pronounced protection against thermal denaturation afforded by MB, and Fig. 2B shows Differential Scanning Calorimetry (DSC) scans in the absence and presence of the ligand [12]. From these scans it can be calculated that in the MB/*Tc*AChE complex, relative to the free enzyme, there is a significant increase in the activation energy of denaturation, E_A (from 74.4 to 146.8 kcal mol^{-1}), and the transition temperature also increases (from 42.6 to 47.9 °C). Furthermore, Fig. 2B clearly demonstrates that the cooperativity of the transition is enhanced.

The crystal structure of the MB/*Tc*AChE complex was obtained by soaking the ligand into crystals of the native enzyme followed by data collection at a synchrotron source [12]. The crystal structure reveals a single molecule of MB bound at the PAS, where it is stacked against the conserved tryptophan residue, Trp279. However, it is oriented along the gorge, towards the active site, in keeping with the observation that the CAS inhibitor, EDR, can displace it from the active-site gorge (Fig. 3).

2.2. Irreversible inactivation of TcAChE by MB under illumination and biophysical characterization of the photo-oxidized protein

Exposure of *Tc*AChE to illumination by white light in the presence of MB resulted in rapid irreversible inactivation [16]. Inactivation occurred at a similar rate if light at wavelengths below 600 nm was cut off by a filter. When the experiment was conducted in deuterated buffer, pronounced enhancement of the rate of inactivation was observed, supporting a mechanism that involves the action of $^1O_2$ generated by the bound MB [17].

Circular dichroism (CD) spectroscopy of the photo-inactivated *Tc*AChE revealed a slight reduction in ellipticity in the far UV, and somewhat increased binding of the amphiphilic probe, 1-anilino-8-naphthalenesulfonic acid (ANS). These findings indicated that photo-oxidation had produced limited unfolding of the protein. However, both ellipticity in the near UV and intrinsic fluorescence were substantially reduced, suggesting photo-oxidative damage to tryptophan residues [16]. Like other partially unfolded species of *Tc*AChE [18,19], the species generated by photo-oxidation displayed enhanced sensitivity to proteolysis.

In the presence of reversible inhibitors photo-inactivation was retarded, and, conversely, it was shown that photo-oxidation damaged the binding sites for these inhibitors [16].

2.3. Chemical characterization of the photo-oxidized TcAChE

Together with the decrease in intrinsic fluorescence due, apparently, to photo-oxidation of tryptophan residues we observed the

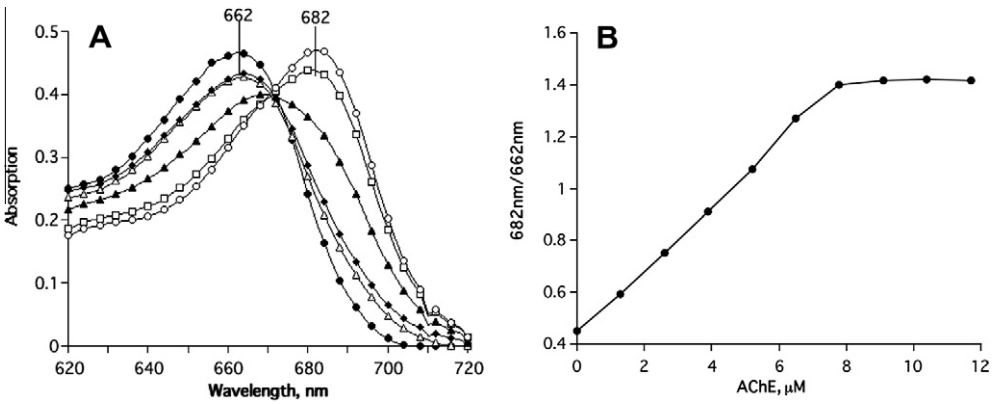


Fig. 1. Monitoring of the bathochromic shift in the absorption maximum of MB produced by its interaction with *Tc*AChE. (A) Shift in the absorption maximum upon titration of 5 μM MB with increasing concentrations of *Tc*AChE; (B) saturation curve calculated on the basis of data similar to those shown in (A) [12].