A spin-labeled derivative of Procion brilliant blue MX-R

Synthesis and interaction with pig heart nucleoside diphosphate kinase

Tudor Porumb, Ioan Lascu*, Horea Porumb†, Radu D. Pop+ and Liliana Bucşa°

*Faculty of Physics, University of Cluj-Napoca, 1, Kogalniceanû Street, R-3400 Cluj-Napoca, Romania, *Department of Biochemistry, †Department of Biophysics and +Department of Physical Chemistry, Medical and Pharmaceutical Institute, Cluj-Napoca, Romania, and °The National Institute for Geriatry and Gerontology, Bucharest, Romania*

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1. INTRODUCTION

The polysulfonated anthraquinone dyes have been extensively used as optical probes for the nucleotide binding sites in dehydrogenases and phosphotransferases [1–6]. Most biochemical studies have been carried out using Cibacron blue 3G-A, as its closely related analogue Procion brilliant blue MX-R reacts covalently with enzymes due to the presence of a reactive chlorine substituent (fig.1) [7]. The belief that Cibacron Blue 3G-A binds to enzymes possessing the supersecondary structure called the dinucleotide fold was subsequently shown to have limited value. However, it was highly stimulative for studying the interaction of many proteins with anthraquinone dyes.

This paper presents the synthesis of a spin label probe SL-Procion by the reaction of Procion brilliant blue MX-R with TEMPO-amine. The probe has approximately the same size as Cibacron blue 3G-A, but lacks one negative charge. Its interaction with NDP kinase was examined and compared to that of Cibacron blue 3G-A.

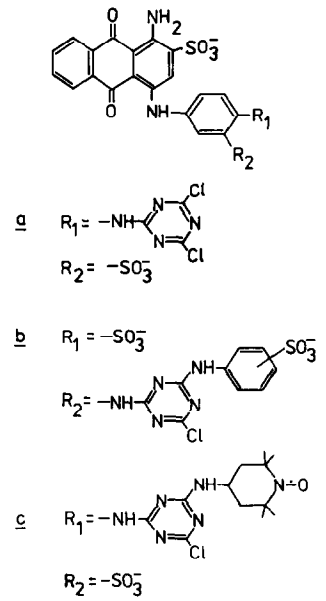

Fig.1. Chemical formulae of (a) Procion brilliant blue MX-R, (b) Cibacron blue 3G-A and (c) SL-Procion.

Abbreviations: SL-Procion, the spin-labeled derivative resulting from the substitution of a chlorine atom from the triazine ring of Procion brilliant blue MX-R (Colour Index generic name Reactive Blue 4, Constitution no.61205) by 4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl (fig.1c); TEMPO-amine, 4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl; NDP kinase, nucleoside triphosphate–nucleoside diphosphate phosphotransferase (EC 2.7.4.6)

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2. MATERIALS AND METHODS

2.1. *Chemicals and analytical procedures*

Procion brilliant blue MX-R was a product of ICI (a kind gift of Dr I.S. Ishimaru, Osaka, Japan). TEMPO-amine was synthesized as in [8,9]. NDP kinase was purified from pig hearts to apparent homogeneity by affinity chromatography as in [10]. Protein concentrations were measured using $A^{1\%}_{280} = 12.6$ [5]. In all calculations the molar concentration of NDP kinase was expressed as subunit concentration assuming an M_r of 17000 [10].

The kinetic, spectroscopic and polarographic analyses were carried out as in [5]. The ESR solution spectra were recorded at X-band frequency using a conventional ART-6 spectrometer (IFTM, Bucharest), with 100 kHz modulation, in capillary tubes, at 25°C.

The concentrations of Procion brilliant blue MX-R and of SL-Procion were determined spectrophotometrically assuming an absorbance coefficient of 13.6 $mM^{-1} \cdot cm^{-1}$ at 610 nm, the same value as for Cibacron blue 3G-A, which has the same chromophore [11].

2.2. *Synthesis of SL-Procion*

TEMPO-amine and Procion brilliant blue MX-R were purified prior to use by thin-layer chromatography (TLC) as described below for the purification of the reaction product. Procion brilliant blue MX-R yielded a major band and at least 5 coloured minor bands. The reaction was carried out in water–ethanol (80:20, v/v). The reaction mixture contained 100 μmol Procion brilliant blue MX-R, 170 μmol TEMPO-amine and 500 μmol sodium bicarbonate in a volume of 4 ml. The reaction mixture was sealed and plunged in a thermostatted water bath for incubation at 35°C for 30 min or left overnight at room temperature.

The product and unreacted components were separated by TLC on 10 × 10 cm silica gel 60 F_{254} plates (Merck, Darmstadt), about 50 μl reaction mixture/plate, with tetrahydrofuran–water (15:1, v/v) as eluent. The product was recovered by scratching the silica layer and elution with a small volume of distilled water. The reaction product (R_f = 0.36) is readily separated from the unreacted Procion brilliant blue MX-R (R_f = 0.25) and unreacted TEMPO-amine (R_f = 0.12). Under the reaction conditions described above the conversion of Procion brilliant blue MX-R to its spin label derivative was complete. The derivative was stable in aqueous solution (about 1 mM) frozen at −12°C in the dark, with no sign of alteration (change in R_f or loss of spin), for at least 3 months.

3. RESULTS

3.1. *Characterization of the reaction product*

The visible absorption spectrum of SL-Procion in water is identical with that of Procion brilliant blue MX-R. Procion brilliant blue MX-R and SL-Procion at neutral pH reduce polarographically at −0.58 V, a value close to that obtained for Cibacron Blue 3G-A [5].

The ESR spectrum of SL-Procion in aqueous solution shown in fig.2a is characteristic of nitroxide radicals freely tumbling in solution [12]. The unequal broadening of the 3 hyperfine lines is due to a decrease of the rotational reorientational rate of the molecule because of the considerably large Procion blue MX-R moiety and/or formation of aggregates. The free TEMPO-amine in solution yields 3 approximately equal lines, similar to those shown in fig.2c.

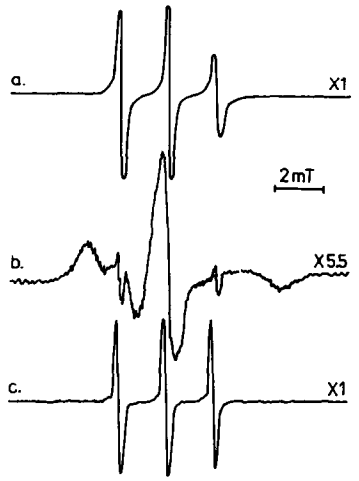

Fig.2. X-band ESR spectra of (a) 0.53 mM SL-Procion in 20 mM phosphate buffer (pH 7.0); (b) 0.525 mM SL-Procion complexed with 1.73 mM NDP kinase in 20 mM phosphate buffer (pH 7.0), containing 1 mM EDTA; (c) 0.53 mM TEMPO-amine in the presence of excess NDP kinase, in the same buffer as for (b). The amplitude of the 100 kHz modulation field was 0.1 mT in all cases.

3.2. *Binding of SL-Procion to NDP kinase studied by ESR*

The tight binding of SL-Procion to NDP kinase is demonstrated by the ESR spectrum of the complex (fig.2b), the line shape of which is characteristic of immobilized (on the ESR time scale) nitroxide radicals [12]. In control experiments it was shown that TEMPO-amine does not bind to NDP kinase (fig.2c).

A contribution from a mobile (unbound) component is also apparent in the spectrum from fig.2b. This contribution is enhanced by at least one order of magnitude in terms of heights of the corresponding features in the spectrum, because of the narrow hyperfine structure characteristic of the mobile (unbound) SL-Procion species. The proportion can be inferred from examination of the spectra from fig.2a,b, and was reproduced theoretically by synthesis of the line shapes (not shown) [13].

4. DISCUSSION

The high reactivity of the chlorine atom from dichlortriazine derivatives allows the use of mild conditions for the coupling of such derivatives to polysaccharide substrates [14]. For the same reason, the use of these dyes as optical probes is limited, as they react with reactive groups in proteins [7]. It is thus expected that the synthesis of derivatives by substitution of one of the chlorine atoms from the triazine nucleus proceeds readily with nucleophilic reagents. This may be a simple way for insertion of photolabile ($-N_3$) or fluorescent groups or of radioactive labels (^{125}I). Here we describe the synthesis of a spin-labeled derivative.

The ESR experiments demonstrate the usefulness of this derivative as a spin probe. The appearance of a strongly immobilized type spectrum (fig.2b) in the presence of NDP kinase is a consequence of the loss of rotational freedom of the spin label group upon binding of the derivative to the enzyme. Since the inhibition of the enzyme activity by SL-Procion is competitive with respect to ATP (not shown), it is believed that the binding occurs at the nucleoside triphosphate site of NDP kinase. The differential spectrum of the dye bound to protein *vs* free dye is similar to that of the dye in less polar media *vs* the dye in water (not shown). The former is completely abolished upon addition of 175 μM ATP, which is an indication that the SL-Procion binds to the nucleoside triphosphate site even in the absence of Mg^{2+}.

Since the parent dye Cibacron blue 3G-A interferes with the function of many enzymes, supramolecular structures, such as the assembly of tubulin [15], and organelles, such as the mitochondrial transport and phosphorylation of adenine nucleotides [16,17], SL-Procion promises to be a useful probing tool.

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