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Reactivity of Meldola Blue Towards Sulfhydryl Groups: Analytical and Biomedical Aspects

[Meldola Mavisinin Sülfidril Grupları ile Tepkime Yatkınlığı: Analitik ve Biyomedikal Bağlamda Değerlendirmeler]

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ABSTRACT

The reactivity of meldola blue (CI 51175; MB), a phenoxazine redox dye employed in biosensors, towards reduced glutathione (GSH) and protein-SH groups was studied at 25°C in 50 mM MOPS buffer, pH 8.0, containing 25-80 μM MB and (a) GSH (0.25-8 mM), (b) 3-mercaptopropionic acid (3-MPA, 8 mM) or (c) chicken liver sorbitol dehydrogenase (SDH, 40 mU/ml). The reactions were monitored spectrophotometrically at 570 nm (a and b) or 340 nm (c). GSH caused a triphasic change in the spectral properties of MB, pointing to rapid preliminary formation of an adduct (Phase I; K_d = 1.2 mM, r^2 = 0.9994), followed by 2 GSH-dependent (pseudofirst-order) processes (Phases II and III). Phase II was appeared to involve cleavage of the oxazine ring and had a limiting rate constant of 0.45 min^{-1}. Phase III was a slower process with k in the hr^{-1} range. The reaction pattern of the dye with 3-MPA was similar to that with GSH. The reaction with SDH, monitored through the change in enzymatic activity in the presence of 300 mM fructose and 0.2 mM NADH, was fast ($k_{observed} \geq 3.5$ min^{-1} at 80 μM MB) and resulted in inactivation of the enzyme. The results point to the reactivity of MB with SH groups as a significant drawback in dye-mediated analyses using unfractionated biological samples and in amperometric systems based on SH-containing indicator enzymes.

Key Words: Biosensor, meldola blue, sulfhydryl group reactivity, sorbitol dehydrogenase

ÖZET

Biyoalgılayıcı tasarımında kullanılan ve indirgenme-yükseltgenme tepkimelerinde etkili, fenoksazin yapılı bir boya olan meldola mavisinin (meldola blue, CI 51175; MB) indirgenmiş glutatyon (GSH) ve protein-SH gruplarıyla tepkime yatkınlığı, 25°C'de, 50 mM MOPS (pH 8.0) tamponunda incelendi. MB'nin (25 –80 μM) (a) 0.25-8 mM GSH, (b) 8 mM 3-merkaptopropionik asit (3-MPA) ve (c) tavuk karaciğeri sorbitol dehidrogenazı (SDH; 40 mU/ml) ile tepkimeleri, spektrofotometrik olarak izlendi (λ = a ve b sisteminde, 570 nm; c sisteminde 340 nm). GSH, MB'nin spektral özelliklerinde üç aşamalı değişime yol açtı. Hızla gelişen ilk aşamada bir eklenme ürünü oluştuğu (Faz I; K_d = 1.2 mM, r^2 = 0.9994); ilk değişimi, GSH derişimine bağımlı ve görünür birinci dereceden kinetik sergileyen 2 sürecin izlediği (Faz II ve Faz III) görüldü. Hız sabiti üst sınırı 0.45 dak^{-1} düzeyinde olan Faz II sürecinin oksazin halkasının açılmasını yansıttığı düşünüldü. Daha yavaş seyreden Faz III'te k değerleri $saat^{-1}$ boyutundaydı. Boyanın 3-MPA ile tepkime düzeni, GSH tepkimelerine benzer özellikler gösterdi. SDH ile tepkime, enzim etkinliğindeki değişiklik üzerinden izlendi; 300 mM fruktoz ve 0.2 mM NADH varlığında, etkinliğin hızla yok olduğu görüldü. (80 μM MB ile gözlenen k ≥ 3.5 dak^{-1}). Sonuçlar MB'nin SH gruplarıyla tepkime yatkınlığının, ön-kesitleme yapılmamış biyolojik örneklerin analizinde ve SH içerikli algılayıcı enzimlere dayalı boya-aracılı amperometrik düzeneklerde önemli sorunlara yol açabileceğini gösterdi.

Anahtar Kelimeler: Biyoalgılayıcı, meldola mavisi, sülfidril grubu reaktivitesi, sorbitol dehidrogenaz

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INTRODUCTION

Meldola blue (MB; C.I. 51175; Figure 1) is a phenoxazine dye used as a redox mediator in the amperometric detection of a variety of analytes including NADH, dehydrogenase substrates such as NH_4^+ and lactate and specific nucleotide sequences in DNA populations [1-4]. A potential problem in the application of MB-based biosensors to the analysis of heterogeneous samples, though not surfaced so far, is that such biosensors might be subject to interference from reducing contaminants in the sample tested. A relevant contaminant in unfractionated biological material is the sulfhydryl group – present as simple thiols (e.g. glutathione (GSH)) or protein-SH. The aim of the study reported below was to determine the tendency of a selection of SH-containing molecules to reduce MB and thereby to obscure target analyte-related sensor signals. The results of the study indicate that sulfhydryl compounds react with MB, leading to a rapid and complex series of changes in dye structure and that the reactions involving protein-SH are also reflected in changes in protein function.

MATERIALS AND METHODS

Most standard chemicals and biochemicals were purchased from Sigma-Aldrich (USA). D-Fructose and methanol were obtained from Merck (Germany). Sorbitol dehydrogenase (SDH, 0.68 U/mg protein) was partially purified from chicken liveras described elsewhere [5]. Stock solutions of GSH and 3-mercaptopropionic acid (3-MPA) were prepared in 50 mM MOPS buffer (pH 8), just prior to use. Stock solutions of MB (5 mM) were prepared in methanol and renewed daily.

The reactions were carried out at 25°C (in 50 mM MOPS buffer, pH 8) and monitored spectrophotometrically, using a Shimadzu 1601PC spectrophotometer equipped with a Peltier unit.

Reactions of Meldola Blue with GSH and 3-MPA. The reactions were initiated by the addition of 50 μM MB to 0-8 mM GSH or 8 mM 3-MPA in MOPS buffer (v_T = 1 ml). Absorbance was monitored at 570 nm (λ_{max} for MB). With GSH as reactant, overall changes in spectral properties were also recorded (in the 400-700 nm range).

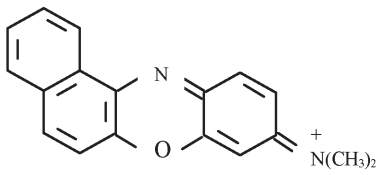
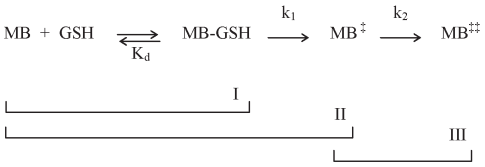


Figure 1. Structure of meldola blue.

The Reaction of Meldola Blue with SDH. The reaction mixture (v_T = 1.2 ml) consisted of 50 mM MOPS (pH 8), containing 300 mM fructose, 0.2 mM NADH, 80 μM MB ± 40 mU SDH/ml. The process was initiated by the addition of MB immediately (within ca. 10 sec) following the addition of enzyme and the consumption of NADH was monitored at 340 nm.

RESULTS

The Reaction of Meldola Blue with GSH and 3-MPA. The reaction of MB with simple sulfhydryl compounds was a triphasic process, as exemplified by the time course of the reaction with GSH (Figure 2). The data were analyzed on the basis of Scheme I, involving preequilibrium formation of an MB-GSH adduct (Phase I), followed by rate-limiting conversion to $MB^{\ddagger}$ (Phase II) and a much slower progress towards a final product (or a mixture of products), $MB^{\ddagger\ddagger}$ (Phase III).



SCHEME I

The equilibrium constant relating to adduct formation in Phase I was determined by using Equations 1-3.

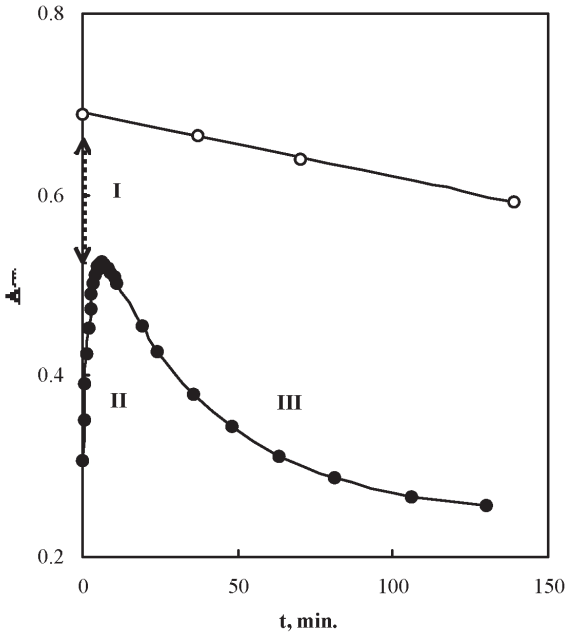


Figure 2. Progress curve for the reaction of meldola blue with GSH. (●), Change in dye absorbance at $[GSH]_o$ = 8 mM, $[MB]_o$ = 50 μM. (o), Control change in absorbance in the absence of GSH.

$$\frac{[MB]_{free}[GSH]_{free}}{[MB\text{-}GSH]} = K_d \quad (1)$$

At $[GSH]_o >> [MB]_o$, $[GSH]_{free} \approx [GSH]_o$ and

$$\frac{[MB\text{-}GSH]}{[MB]_T} = \frac{A_o - A_x}{A_o - A_\infty} = \frac{[GSH]_o}{K_d + [GSH]_o} \quad (2)$$

(A_o and A_x = "zero-time" absorbance readings in the presence of 0 and x mM GSH, respectively. A_∞ = "zero-time" absorbance reading at saturating [GSH]).

$$\frac{1}{A_o - A_x} = \frac{K_d}{(A_o - A_\infty)[GSH]_o} + \frac{1}{A_o - A_\infty} \quad (3)$$

Double reciprocal plots of $1/(A_o - A_x)$ versus $1/[GSH]_o$ were linear (Figure 3) and yielded K_d = 1.2 mM (r^2 = 0.9994).

Phases II and III were analyzed by treating the formation of $MB^{\dagger}$ and $MB^{\dagger\dagger}$ as a consecutive process (Equations 4-6) [6].

$$d[MB^{\dagger}]/dt = k_1[MB\text{-}GSH] = k_1\{[GSH]/(K_d + [GSH])\}[MB]_{T,t} \quad (4)$$

$$k_1' = k_1\{[GSH]_o/(K_d + [GSH]_o)\} \quad (4a)$$

$$d[MB^{\dagger\dagger}]/dt = k_2[MB^{\dagger}]_t \quad (5)$$

$$[MB^{\ddagger}]_t = \frac{k_1'[MB\text{-}GSH]_o}{(k_2 - k_1')}(\exp[-k_1't] - \exp[-k_2t]) \quad (6)$$

The exponentials were resolved graphically by replacing concentration terms with A_{570} values and constructing semilogarithmic plots of ln $(A_t - A_\infty)$ versus time (Figure 4). The biphasic plots so obtained were analyzed as described in [6]: The linear segment of the plot (corresponding to the time interval in which the first exponential term in Eq. 6 was negligible) yielded - k_2 as slope. The linear segment was extrapolated to t = 0; k_1' was

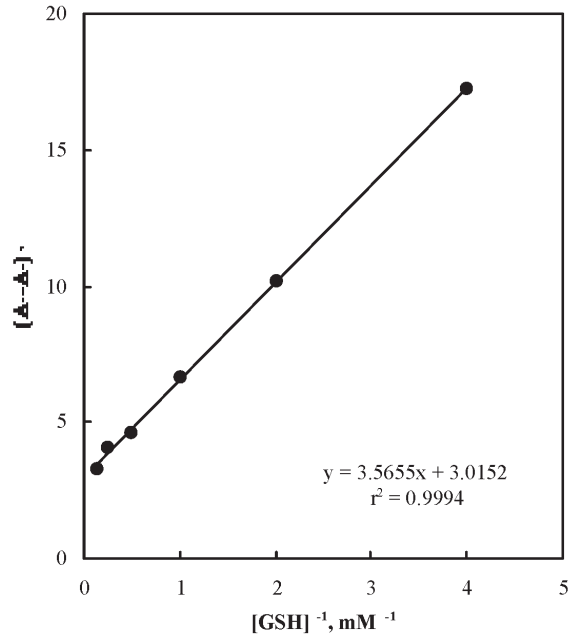
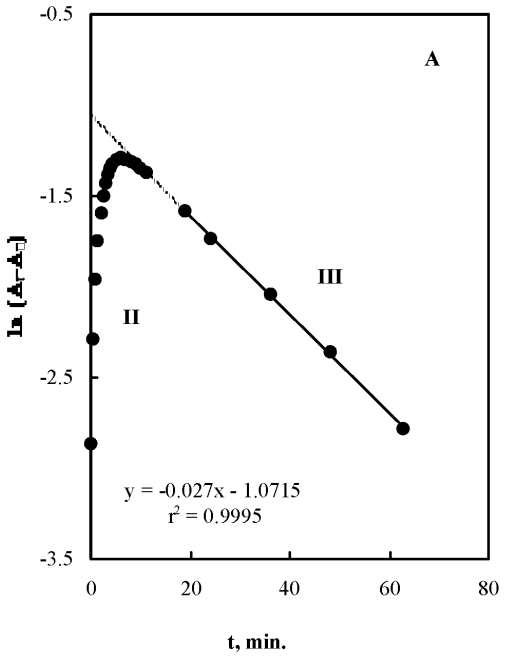


Figure 3. Dependence of the amplitude of Phase I in the reaction of GSH with MB on $[GSH]_o$. $[MB]_o$ = 50 μM.



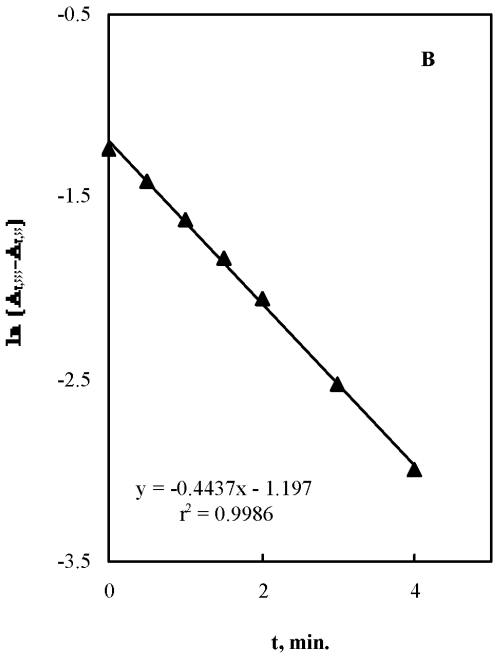


Figure 4. Semilogarithmic progress curves for the reactions of the MB-GSH adduct.

$[GSH]_o$ = 8 mM, $[MB]_o$ = 50 μM.

(A) Overall progress curve. The linear segment spans the time interval where the first exponential term in Eq. 6 (see text) is negligible. The slope of this segment (Phase III) = - k_2'. (B) Semilogarithmic replot of the difference between $(A_t - A_\infty)$ in the virtual segment of Phase III (Fig. 4A, dashed line) and the observed values of $(A_t - A_\infty)$ in Phase II (Fig. 4A, ascending curve). The slope of this plot = - k_1'.

obtained from a semilogarithmic replot (Fig. 4B) of the difference between (A_t - A_∞) in the virtual segment of Phase III (Fig. 4A, dashed line) and the observed values of (A_t - A_∞) in Phase II (Fig. 4A, ascending curve).

As predicted by Scheme I (and Eq. 4a), k_1' showed a hyperbolic dependence on $[GSH]_o$, reaching a limiting value (k_1) of 0.45 min^{-1} (not shown). Phase III was also a [GSH]-dependent process (Figure 5). The linear dependence of the observed value of k_2 (k_2') on [GSH] most likely arose from a catalytic involvement of GSH in the conversion of $MB^{\dagger}$ to $MB^{\dagger\dagger}$. The inherent value of k_2, estimated from the ordinate intercept in Fig. 5 was ca. 1 x 10^{-3} min^{-1}.

The spectral changes accompanying Phases I-III in the reaction of GSH with MB are given in Figure 6. The reaction of 3-MPA with MB had a pattern similar to that observed with GSH, except that k_2' was independent of [3-MPA].

The Reaction of Meldola Blue with SDH. The reaction between MB and SDH was studied by monitoring the time-dependent change in enzyme activity, as reflected in the progress curve for NADH depletion under standard assay conditions. The capacity of MB to oxidize NADH was a potential problem to consider, since the observed enzymatic rate could change not only because of a direct effect of the dye on protein structure and activity, but also because of a significant change in [NADH] relative to K_{NADH}. Preliminary experiments showed that, at high $[fructose]_o$ values, the rate of the SDH-catalyzed conversion of fructose to sorbitol remains constant down

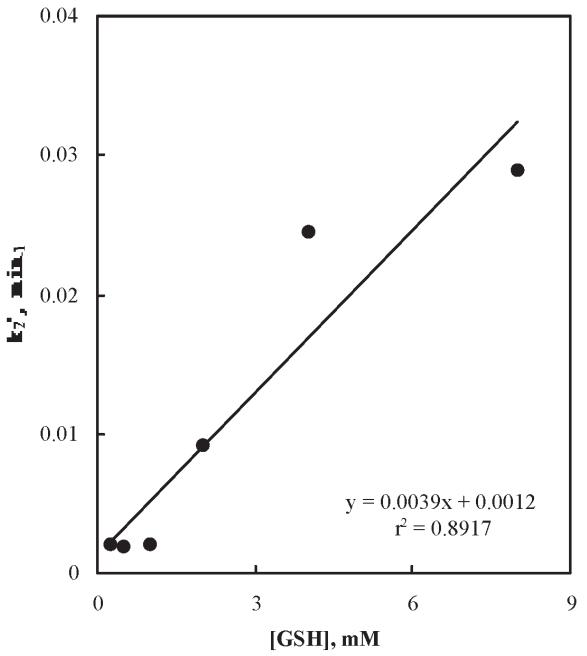


Figure 5. The dependence of the observed rate constant for Phase III (k_2') on $[GSH]_o$.

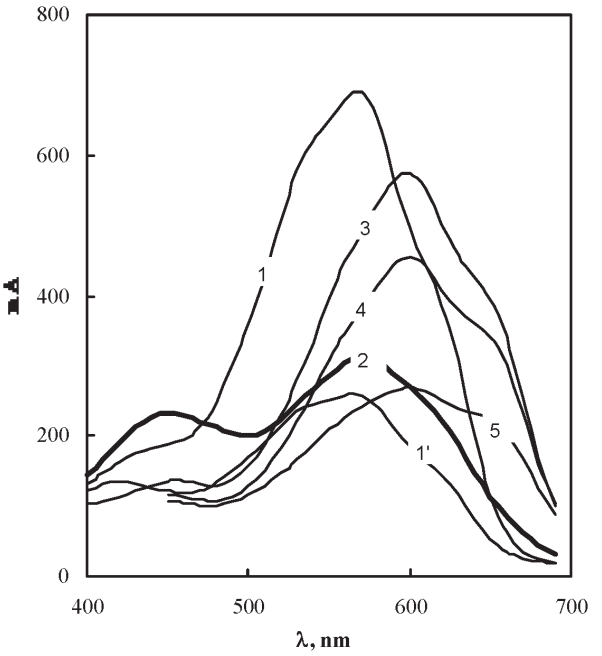


Figure 6. Spectral changes relating to the reaction between MB and GSH.

[GSH] = 8 mM, [MB] = 50 μM

1 and 1': Control spectra at t = 0 and after overnight incubation.

2-5: Spectra recorded at t = 0, 5, 30 and 110 min. following the addition of GSH. (Cf Fig. 2).

to ≈ 10 μM NADH [7]. Hence it was feasible to use enzymatic progress curves in the presence and absence of MB in evaluating the direct impact of the dye on the SDH molecule. The relevant progress curves are given in (Figure 7). The control curve reflects the oxidation of NADH by MB; the absorbance change in the experimental curve is the combined result of NADH oxidation by MB and the NADH-coupled SDH-catalyzed reduction of fructose. The control curve leveled out at ca. 70 μM NADH. (This value was significantly lower than that expected from a stoichiometric reaction between cofactor and dye, as $[NADH]_o$ - $[MB]_o$ ≈ 120 μM. The reason for the discrepancy remains to be accounted for). The critical feature of the experimental curve was that the lower limit of [NADH] was the same as in the control curve. This indicated that SDH was inactivated by MB in the interim period, since the inherent enzymatic rate in the absence of MB (and the initial rate in the presence of MB) amounted to 0.24 absorbance units/min. The reaction conditions employed in this preliminary study do not allow quantitative analysis of the inactivation process. However, the total loss of SDH activity in the 0-60 sec segment of the experimental curve (Figure 7), indicates that the half-life of the enzyme in the presence of dye must be ≤ 12 sec.

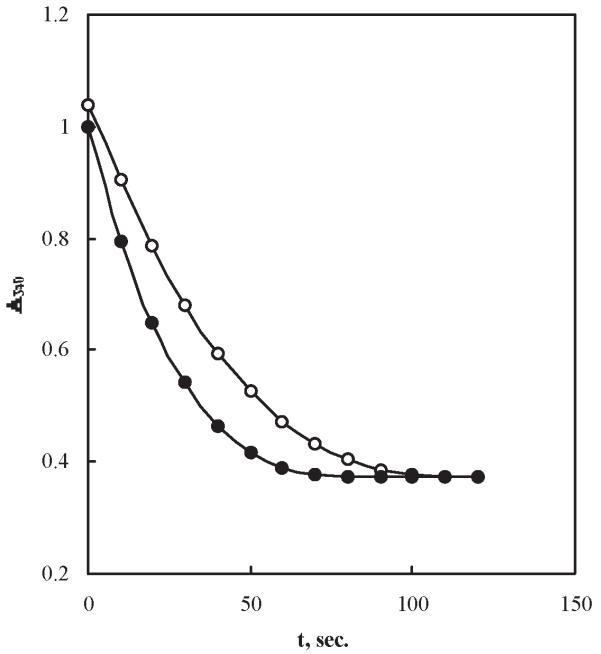
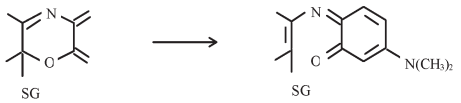


Figure 7. Time course of NADH depletion in the presence of MB ± SDH.

(o), Control reaction of NADH with MB; (●) depletion of NADH in the presence of MB and SDH. [NADH] = 0.2 mM, [MB] = 80 μM, [SDH] = 40 mU (ca. 60 μg protein)/ml.

DISCUSSION

The results presented showed MB to react with simple thiols to give adducts which proceed to yield at least one thiol-related secondary product. The initial, fast adduct formation (Phase I) was similar to that observed with glutathione and the cationic triarylmethane dyes (TAMs), malachite green and methyl green, where K_d has been estimated at 0.2 and 0.02 mM, respectively [8]. The subsequent Phase II appeared to involve an intramolecular conversion and had no counterpart in the GSH-TAM system. While the exact nature of the intramolecular process is unknown at this point, it may be speculated that it involves cleavage of the oxazine ring (Scheme II).



SCHEME II

The fast depletion of the parental dye molecule by GSH (and 3-MPA) bore out the predicted problem concerning the use of MB as a redox mediator in biological sample analysis. The inactivation of SDH, a sulfhydryl-containing enzyme, further showed that biosensors incorporating enzymes may suffer from instability due to depletion of their catalytic content. This problem has recently been discussed in relation to the shelf-life of commercial, dehydrogenase-based electrodes for monitoring of 3-hydroxybutyrate [9].

It would be of interest to determine to what extent different phenoxazines (and phenothiazines) share the SH-reactivity of MB, as such compounds are under investigation as chemosensitizers and preventive agents in cancer and chemotherapy [10-11].

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