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This paper is dedicated to Professor Włodzimierz Ostrowski

Continuous assay for acid phosphatase using 1-naphthyl phosphate as a substrate*

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The described continuous acid phosphatase assay is based on kinetics of the release of 1-naphthol in the course of the enzyme-catalyzed hydrolysis of 1-naphthyl phosphate, measured at 320 nm in aqueous solution and at 322 nm in sodium-bis(2-ethylhexyl)sulfosuccinate isooctane-water reverse micelles in a broad pH range (1.0–8.2). The method allows precise determination of the initial rate of the reaction and therefore may be used in the steady-state and pre-steady-state studies on the phosphatase-catalyzed reaction. The kinetic parameters (K_m and k_{cat}) for human prostatic acid phosphatase in aqueous solution and in reverse micelles, at pH 3.8, 4.5 and 5.7, by the proposed 1-naphthyl phosphate assay have been determined.

Acid phosphatases (EC 3.1.3.2) are non-specific enzymes catalyzing the hydrolysis of low and high molecular phosphomonoesters in an acidic environment [1]. Human prostatic acid phosphatase (hPAP) is of special interest and is routinely assayed in diagnosis and monitoring of patients with prostatic carcinoma [2–4]. For determination of acid phosphatase activity various phosphomonoesters were used and the released reaction products were measured in a discontinuous or continuous manner [1–4]. In discontinuous procedures and in coupled assays the equilibrium of the phosphatase-catalyzed reaction is disturbed and only continuous methods enable precise determination of kinetic constants of the enzyme-catalyzed reaction due to monitoring of the reaction progress.

Since 1959 1-naphthyl phosphate has been used by enzymologists and clinical chemists as a substrate for acid phosphatase in the discontinuous or coupled assays. For colorimetric or spectrofluorimetric determination of phosphate [5] or 1-naphthol [6–18] many procedures were used. Formation of a stable and reproducible colour of 1-naphthol with aminoantipyrine and ferricyanide is utilized in quantitative measurements of phosphatase activity [6]. Babson's procedure is based on the measurement of azo dye

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Abbreviations: AOT, sodium-bis(2-ethylhexyl)sulfosuccinate (Aerosol OT); w_o, water to detergent molar ratio ($w_o = [H_2O]/[AOT]$); hPAP, human prostatic acid phosphatase.

arising in the reaction of 1-naphthol with tetrazotized *O*-dianisidine [7, 8]. Basing on the same principle the use of Fast Red Salt B (diazotized 5-nitro-2-amino-methoxybenzene) [9], of Fast Red TR (diazotized 2-amino-5-chloro-toluol) [10, 11], and of some other diazonium salts [12] was recommended for acid phosphatase assays in serum [13]. After alkalization of the sample, concentration of 1-naphthoxide ion is determined in the endpoint procedure by measuring its absorbance at 332 nm [13] or spectrofluorimetrically at 455 nm (λ_{exc} 335 nm) [14]. 1-Naphthyl phosphate was also used as a substrate for staining of acid phosphatase activity in histochemical studies [15], in gel electrophoresis [16, 17] and in sodium-bis(2-ethylhexyl)sulfosuccinate (Aerosol OT)-isooctane-water reverse micelles in the coupled reaction with Fast Red [18]. Until now no continuous one-step procedure for acid phosphatase assay using 1-naphthyl phosphate has been proposed.

A direct continuous spectrophotometric method for determination of the phosphatase-catalyzed hydrolysis of 1-naphthyl phosphate is described in this paper. The assay is based on kinetics recording of the release of 1-naphthol by measuring the difference of 1-naphthol and 1-naphthyl phosphate absorbance at 320 nm in aqueous solution, or at 322 nm in AOT-isooctane-water reverse micelles at pH 1.0 to 8.2.

Reverse micelles, water microdroplets dispersed in apolar solvents and stabilized by monolayer of surfactant, are optically transparent and can be examined with various optical instruments. The size of these spheroidal microaggregates depends on the water-to-surfactant (w_o) molar ratio and is comparable to protein molecule dimensions. The micellar inner interface and the aqueous phase provide a unique and versatile environment for the study of biocatalysis by housing a guest enzyme together with its substrate [19–21].

MATERIALS

Homogeneous human prostatic acid phosphatase was purified from human seminal plasma [22]. Phosphatase concentration was determined by measuring the absorbance at 280 nm ($A^{1\%}_{280}$ = 14.4) (in 10 mM Tris/HCl buffer, pH 7.4, containing 100 mM NaCl) [2].

Disodium salt of 1-naphthyl phosphate and sodium salt of succinate acid bis(2-ethylhexyl)ester (AOT) were purchased from Sigma. 1-Naphthol from POCH (Poland) was recrystallized from water. Isooctane for UV spectroscopy, sodium acetate, acetic acid, and Tris were from Fluka.

METHODS

Spectrophotometric assays. Absorbance spectra and velocity measurements were recorded at 20°C using the single beam UV-VIS Gillford Response Spectrophotometer.

Buffers. The 50 mM acetate (pH 3.8–5.7) and Tris/HCl (pH 7.2–9.2) buffers in deionized and distilled water were used.

Continuous enzyme asssay in aqueous solution. Determination of phosphatase activity was started by adding 0.05 ml of the enzyme solution (diluted to the required concentration from the concentrated enzyme stock solution) into 0.95 ml of 1-naphthyl phosphate solution of appropriate concentration in 50 mM acetate buffers of different pH. In the blank sample, the enzyme was omitted. The assay was conducted at 20°C. The acid phosphatase-catalyzed hydrolysis of 1-naphthyl phosphate in acidic solution was monitored continuously by measuring the increase of 1-naphthol absorbance at 320 nm. Initial rates were determined by the Gillford Spectrophotometer program. In the conditions used, the spontaneous substrate hydrolysis could be neglected.

Reverse micellar solution. AOT-isooctane water reverse micelles were formed by injection of microlitre amounts of aqueous buffered stock solution of either enzyme or substrate to 1 ml of 0.1 M AOT solution in isooctane and vortexed. For a particular w_o the required volume of aqueous solution was calculated as described elsewhere [23].

Continuous enzyme assay in AOT-isooctane-water reverse micelles. The assay was performed by combining 0.5 ml of the substrate micellar solution with 0.5 ml of the

enzyme micellar solution prepared under the same conditions (buffer, pH, w_o) and stirring using a warring blender. The assay was started immediately at 20°C with continuous monitoring of 1-naphthol absorbance at 322 nm. The initial velocity was calculated by the Gillford Spectrophotometer software. The overall concentrations of all reagents in reverse micelles were marked ov.

Calibration curves. The standard curves for 1-naphthol were constructed in aqueous solution at 320 nm and in reverse micelles at 322 nm. The molar absorption coefficients were calculated by the Gillford Spectrophotometer software.

Determination of kinetic constants for hPAP. The values of K_m and k_{cat} in aqueous solution for each pH value, at the enzyme concentration of 9.25 nM, were calculated [24] from triplicate measurements of v_o for 1-naphthyl phosphate concentration in the range from 0.05 mM to 5 mM.

For determination of $K_{m,ov}$ and k_{cat} [24] in AOT-isooctane-reverse micelles, the initial velocity (v_o) measurements in the micellar solution at w_o 20 for each pH were performed in triplicate. The concentration of the substrate and the enzyme was as in aqueous solution.

RESULTS

Absorbance spectra of 1-naphthyl phosphate and of 1-naphthol in aqueous solution and in AOT-isooctane-water reverse micelles

The spectra of 1-naphthyl phosphate and of 1-naphthol at pH 1.0–8.2 are essentially different (Fig. 1A, 1B). At 320–322 nm the absorbance of 1-naphthyl is high and of 1-naphthyl phosphate — low. Therefore 1-naphthol, liberated in the enzyme-catalyzed hydrolysis of 1-naphthyl phosphate, was measured at 320 nm in aqueous solution and at 322 nm in reverse micelles (Fig. 1C).

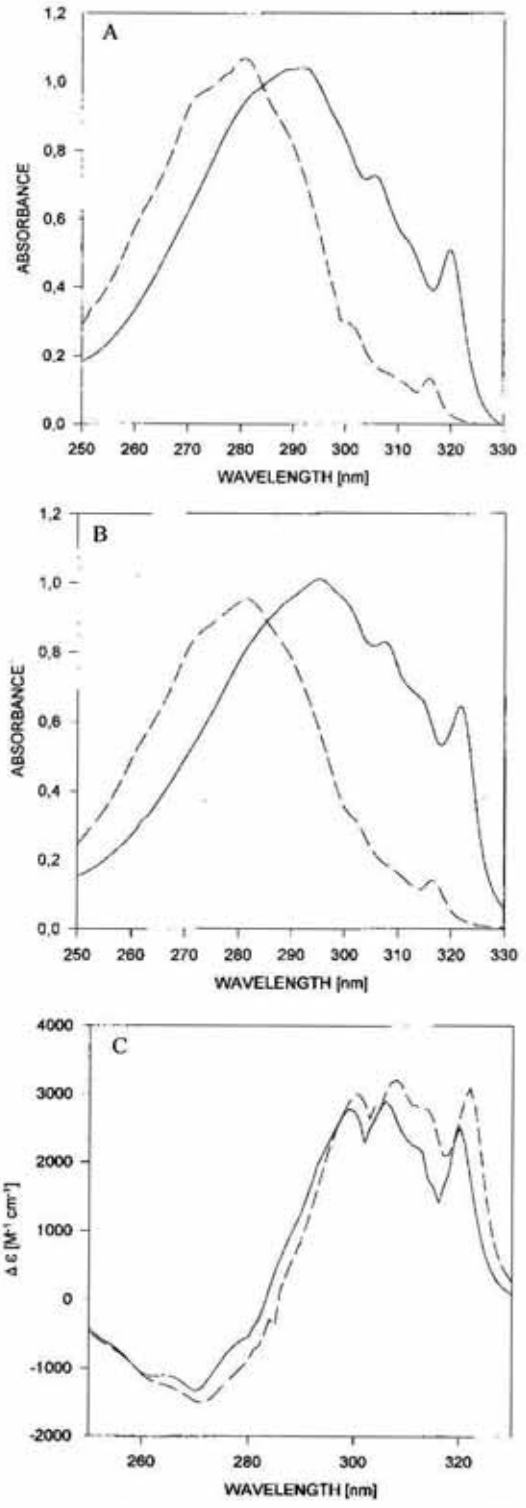

Figure 1. Absorption spectra of 0.2 mM 1-naphthol (—) and of 0.2 mM 1-naphthyl phosphate (- - -) in 50 mM acetate buffer at pH 4.5. A, in aqueous medium; B, in AOT-isooctane-water reverse micelles, w_o 20; C, the difference in the molar absorption coefficients of 1-naphthol and 1-naphthyl phosphate in aqueous solution and in reverse micelles (- - -), at pH 4.5.

Molar absorption coefficients for 1-naphthol and for 1-naphthyl phosphate in aqueous solution and in AOT-isooctane-water reverse micelles

The calibration curves for 1-naphthol in aqueous solution are linear up to 1 mM and in reverse micelles up to 0.1 mM. The molar absorption coefficients for 1-naphthol are constant at pH up to pH 8.2 (pK 9.38 [25]) and for 1-naphthyl phosphate up to pH 4.8 (pK 5.85, [26]).

The molar absorption coefficients for 1-naphthol at pH range from 1.0 to 8.2 equals 2600 M^{-1} cm^{-1} at 320 nm in aqueous solution, and 3100 M^{-1} cm^{-1} at 322 nm in AOT-isooctane-water reverse micelles (w_o 10 to 30).

The molar absorption coefficients of 1-naphthyl phosphate in aqueous solution at 320 nm are: 175 M^{-1} cm^{-1} (pH 1.0–4.5), 520 M^{-1} cm^{-1} (pH 5.7) and 825 M^{-1} cm^{-1} (pH 7.3–13.0) and in AOT-isooctane-water reverse micelles (w_o 10 to 30) at 322 nm are: 200 M^{-1} cm^{-1} (pH 1.0–4.5), 300 M^{-1} cm^{-1} (pH 5.7) and 450 M^{-1} cm^{-1} (pH 7.3–13.0).

The difference between molar absorption coefficients ($\Delta\varepsilon$) of 1-naphthol and of 1-naphthyl phosphate at 320 nm in aqueous solution are as follows: 2425 M^{-1} cm^{-1} (pH 1.0–4.5), 2080 M^{-1} cm^{-1} (pH 5.7) and 1775 M^{-1} cm^{-1} (pH 7.3–8.2) and in reverse micelles at 322 nm are: 2900 M^{-1} cm^{-1} (pH 1.0–4.5), 2800 M^{-1} cm^{-1} (pH 5.7) and 2650 M^{-1} cm^{-1} (pH 7.3–8.2).

Kinetic parameters of human prostatic acid phosphatase in aqueous solution and in AOT-isooctane-water reverse micelles

Kinetic parameters for hPAP at pH 3.8, 4.5 and 5.7 in aqueous solution (K_m and k_{cat}) and in AOT-isooctane-water reverse micelles ($K_{m,ov}$ and k_{cat}) with 1-naphthyl phosphate as a substrate measured by the continuous procedure, are given in Table 1.

DISCUSSION

Continuous acid phosphatase assays developed earlier [5, 27–34] have employed the numerous phosphomonoesters: *O*-carboxyphenyl phosphate [5], phenyl phosphate [27], phosphotyrosine [28, 29], 2,6-dichloro-4-nitrophenyl phosphate [30, 31], *p*-nitrophenyl phosphate [25, 32, 33], 4-methylumbelliferyl phosphate [25] and pyranine phosphate [34]. The 1-naphthyl phosphate continuous acid phosphatase assay in aqueous solution proposed in this paper is more sensitive than that with phenyl phosphate [27], phosphotyrosine [28, 29] or *p*-nitrophenyl phosphate (below pH 6.0) [32, 33], but is of almost the same sensitivity as with *O*-carboxyphenyl phosphate [5] or 4-methylumbelliferyl phosphate [25], as shown by molar absorption coefficients of the phenolic reaction products.

Besides the coupled 1-naphthyl phosphate procedure of Levashov *et al.* [18], two continuous acid phosphatase assays in triphasic AOT-isooctane-water reverse micelles has been developed earlier, using as substrates *p*-nitrophenyl phosphate [35] and phenyl phosphate [27]. However, the continuous 1-naphthyl phosphate assay in the same medium described in this paper, is more sensitive than the continuous assay using phenyl phosphate [27] as a substrate.

The values K_m of hPAP in aqueous solution (Table 1) are at lower range of those obtained earlier ($0.9–9.8 \times 10^{-4}$) by the various discontinuous methods in different buffers and with

Table 1. Kinetic constants for human prostatic acid phosphatase in 50 mM acetate buffer, using 1-naphthyl phosphate as a substrate or entrapped in 0.1 M AOT-isooctane-water reverse micelles (w_o 20)

Buffer	Aqueous solution		Micellar solution	
pH	K_m [M]	k_{cat} [s^{-1}]	$K_{m,ov}$ [M]	k_{cat} [s^{-1}]
3.8	1.8×10^{-4}	850	7.4×10^{-4}	80
4.5	1.6×10^{-4}	1000	7.7×10^{-4}	90
5.7	1.4×10^{-4}	1110	7.5×10^{-4}	50

different additives [26, 36–39]. The values of k_{cat} have never been determined directly, only the V_{max} values were given [26, 36, 39].

The values of $K_{m,ov}$ for the hPAP-catalyzed hydrolysis of 1-naphthyl phosphate in AOT-isooctane-water reverse micelles are somewhat higher than those in aqueous medium (Table 1), but are still of the same order. On the other hand, the k_{cat} values in reverse micelles are very low in comparison to those in aqueous medium. The inhibition of hPAP in the AOT-isooctane-water reverse micelles can be due to the interaction of the positively charged groups at the active site of the enzyme molecules [3], with the negatively charged AOT molecules at the inner surface of reverse micelles. Accumulation of phosphate ion, a known inhibitor of phosphatases [2], inside the polar pool of the micelle may be an additional reason for the decrease of k_{cat} values for hPAP entrapped in reverse micelles. When pH is raised from 3.8 to 5.7, the k_{cat} values in aqueous medium increase, whereas in the micellar surrounding they decrease. An inhibition of the enzyme activity in the AOT-isooctane-water reverse micelles was also found in the case of wheat germ acid phosphatase [18], human prostatic acid phosphatase [27], human placental alkaline phosphatase [40], α-chymotrypsin [23] and lactate dehydrogenase [41].

The proposed assay as a sensitive continuous procedure may be especially useful for the pre-steady-state studies at a broad pH range (1.0 to 8.2) of various phosphatases .

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