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2-Methoxybenzoyl phosphate: a new substrate for continuous fluorimetric and spectrophotometric acyl phosphatase assays

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Abstract. A new aromatic acyl phosphate, 2-methoxybenzoyl phosphate, has been synthesized. The compound shows an intrinsic fluorescence; it displays an intense emission band at 390 nm upon excitation in the near UV region. This band practically disappears after hydrolysis of the product. On the other hand, the product displays differences in the near UV absorption spectra measured before and after hydrolysis. The $\Delta\varepsilon$ at 301 nm is 2720 M^{-1} cm^{-1}, a value that is 4.3-fold higher than that of benzoyl phosphate (the usual substrate for acylphosphatase assay) at 283 nm. The main kinetic parameters of three different acylphosphatase molecular forms (the muscular isoenzyme and two subtypes of the organ common isoenzyme) were determined using both benzoyl phosphate and 2-methoxybenzoyl phosphate as substrates, and then compared. These kinetic data and the UV absorption and fluorescence properties of 2-methoxybenzoyl phosphate suggest that this compound has better substrate features than benzoyl phosphate, and can be used for both high sensitivity continuous fluorimetric and UV absorption spectrophotometric assays of acylphosphatase.
Key words. 2-methoxybenzoyl phosphate; acylphosphatase.

Acylphosphatase is a low M_r enzyme expressed in all vertebrates. Two isoenzymes, coded for different genes, are differentially expressed in several tissues[1,2]. The muscle type isoenzyme accounts for most acylphosphatase activity of skeletal muscle and heart, whereas the organ common type isoenzyme is highly expressed in tissues such as brain, testis and erythrocytes. The muscle type was first isolated[3] and sequenced[4] in our laboratory. Later, the first organ common type isoenzyme was purified and then its primary structure was determined[5]. The enzyme was also isolated from several different tissues and species and sequenced (ref. 6, and citations herein). The complete three-dimensional structure of the muscle type isoenzyme in solution was determined by NMR techniques[7–10]. Acylphosphatase has been implicated in the control of ionic conditions of the intracellular medium, since it catalyzes the hydrolysis of the phosphoprotein intermediate formed in the action of the membrane Na^+, K^+ and Ca^{2+} pumps[11–17]. Altered acylphosphatase levels have been demonstrated in hyperthyroid and Duchenne muscular dystrophy patients[18,19], as well as in tissues of rats treated with tri-iodothyronine[20]. This suggests a potential clinical use for the acylphosphatase assay.

This paper deals with the synthesis and optical properties of 2-methoxybenzoyl phosphate (2MBP)†. This substrate was selected from several synthetic acyl phosphates since it displays fluorescence properties and other characteristics that render it useful for kinetic studies on acylphosphatase isoenzymes. In addition this substrate is applicable to high sensitivity acylphosphatase assay in cell culture extracts.

Materials and methods

Triethylamine, 2-methoxybenzoic acid, 2-methoxybenzoyl chloride, and tetrahydrofuran were purchased from Fluka Chemie (Switzerland) Benzoyl phosphate (BP) was synthesized as previously described[21]. The C18 preparative bulk packing chromatographic phase (55–105 μm) was obtained from Waters (USA). The Vydac Protein and Peptide C18 column was purchased from Vydac (USA). All other reagents were the purest commercially available.

Acylphosphatase isoenzymes were prepared from horse skeletal muscle (ACY-M), and from bovine testis (the ACY-1 and ACY-2 organ common subtypes), respectively, as previously described[3,6]. The protein concentrations of the purified enzyme preparations were determined by measuring the UV absorbance, using an extinction coefficient calculated according to the procedure reported by Gill and Hippel[22].

The synthesis of 2MBP was performed as follows: 2-methoxybenzoic acid (40 mmol) was dissolved in 80 ml of tetrahydrofuran, previously chilled in ice, and mixed with 40 mmol of cold triethylamine. Then 40 mmol of 2-methoxybenzoyl chloride were slowly added while stirring. The precipitate formed during the reaction was removed by filtration, and the clarified solution was

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† Abbreviations used: 2MBP, 2-methoxybenzoyl phosphate; BP, benzoyl phosphate; ACY-1, the 98-residue long organ common type isoenzyme of acylphosphatase from bovine testis; ACY-2, the 100-residue long organ common type isoenzyme of acylphosphatase from bovine testis; ACY-M, the muscle type isoenzyme from horse muscle; TFA, trifluoroacetic acid.

concentrated under vacuum maintaining a temperature lower than 20 °C, until a viscous mixture was formed. This, which contains the 2-methoxybenzoic anhydride, was mixed with 48 ml of a solution containing 40 mmol of phosphoric acid dissolved in 30% (v/v) pyridine in water. The mixture was stirred for 30 min at room temperature and then 120 mmol of solid LiCl were added. The product was precipitated by adding 10 volumes of cold acetone, collected by centrifugation, washed with cold acetone, and dried under vacuum over phosphorus pentoxide. The yield of 2-methoxybenzoyl phosphate was about 30%. The product was purified by differential precipitation with acetone, obtaining a 70% pure preparation. The residual impurities, essentially 2-methoxybenzoic acid and Pi, were removed by reverse-phase chromatography on a bulk C18 phase from Waters. A column (2.5 × 30 cm) was first washed with 10 mM acetic acid in methanol, and then equilibrated with 10 mM acetic acid in water. A 120 mM solution of the 70%-pure product was applied to the column and the elution was performed with 10 mM acetic acid in water. Pi eluted first and then 2-methoxybenzoyl phosphate was collected. On the other hand, 2-methoxybenzoic acid remained bound to the column and could be eluted by washing with 10 mM acetic acid in methanol. The fractions containing the product were pooled and lyophilized. Alternatively, the product could be precipitated as disilver salt by adding a molar excess of silver nitrate. The precipitate was collected by filtration, washed with a small volume of distilled water and with cold acetone, and then dried under vacuum over phosphorus pentoxide.

The product was analyzed for acyl phosphate content by the method of Lipmann[23]. This method is based on the very rapid reaction of the acyl phosphate with hydroxylamine to form an acylhydroxamate. This gives a brightly purplish complex in the presence of Fe(III) ions in acidic medium. The calibration curve was determined using 2-methoxybenzoyl chloride. To determine free and organic-bound phosphate, the method of Baginski[24] was performed both before and after 2MBP hydrolysis. The hydrolysis was carried out by incubating a solution of the product in a sealed vial at 100 °C for 30 min. In addition, 2-methoxybenzoic acid was quantitatively determined with reverse phase HPLC, both before and after hydrolysis. The column (Vydac C18) was equilibrated in solvent A (10 mM TFA in a 90/10 (v/v) water/acetonitrile solution). 6 min after sample injection, a gradient of increasing acetonitrile concentration (5% per min) was applied. Under these chromatographic conditions, 2MBP eluted at 4.1 min, whereas 2-methoxybenzoic acid eluted at 13.4 min. A calibration curve was determined by injecting known amounts of standard 2-methoxybenzoic acid.

The absortion spectra were recorded with a Beckman (USA) model 7500 spectrophotometer and the fluorescence emission and excitation spectra were recorded with a Perkin Elmer (USA) model 650-10S spectrophotofluorimeter. The difference absorption coefficients were determined by measuring the absorption spectra before and after hydrolysis of 2MBP buffered solutions. At pH 5.3 (in the apparent pH optimum zone of acylphosphatase, see below) the absorbance difference maximum was found at 301 nm; thus, this wavelength was selected for measurements of acylphosphatase activity by a continuous spectrophotometric test. Similarly, the coefficients relative to the difference in the fluorescence emission were determined by recording the spectra before and after hydrolysis of 2MBP dissolved in 0.05 M sodium acetate buffer, pH 5.3. The excitation at 340 nm was the most useful for fluorescence emission measurements, since at this wavelength the inner filter effect is reduced and the signal was linear over a large concentration range (see below).

K_m and V_{max} relative to acylphosphatase isoenzymes were determined by measuring the initial rates of both 2MBP and BP hydrolyses at different initial substrate concentrations. The concentrations of both substrates were: 1) from 0.05 to 1.5 mM for ACY-1 and ACY-2; 2) from 0.1 to 2.5 mM for ACY-M. They were dissolved in 0.05 M sodium acetate buffer, pH 5.3, and the incubation was carried out at 25 °C. The rates of 2MBP hydrolysis were calculated by following the decrease of both absorbance at 301 nm and fluorescence emission at 390 nm, employing the absorbance and fluorescence coefficients determined as described above. The rates of BP hydrolysis were determined as previously described[25]. The values of K_m and V_{max} were calculated with a non-linear fitting of the hyperbolic Michaelis-Menten equation by using the computer program Fig. P (Fig. P was from Biosoft®, Cambridge, UK.).

The spontaneous hydrolysis of 2MBP at different pH values was measured as follows: 4 mM solutions of the product in suitable buffers, adjusted to different pH values, were prepared. The buffers were: 0.2 M glycine-HCl, pH 2.2; 0.2 M sodium 3,3-dimethylglutarate, pH 3.2 and 5.0; 0.2 M glycine-NaOH, pH 8.6 and 10.4. At pH 12.2, the 2MBP water solution was adjusted to this value by adding sodium hydroxide. Incubations were performed at 25 °C. Aliquots were withdrawn at zero-time and at different interval times; the unhydrolyzed substrate was assayed by the method of Lipmann[23]. The natural logarithm of substrate concentrations at different times was plotted versus time, and the apparent first order kinetic constant was calculated from the best straight line fitting determined by using the Fig. P computer program.

Results and discussion

The product was analyzed for its purity. The reverse phase HPLC analysis of 2MBP preparation is shown in figure 1A, while figure 1B shows the HPLC analysis of

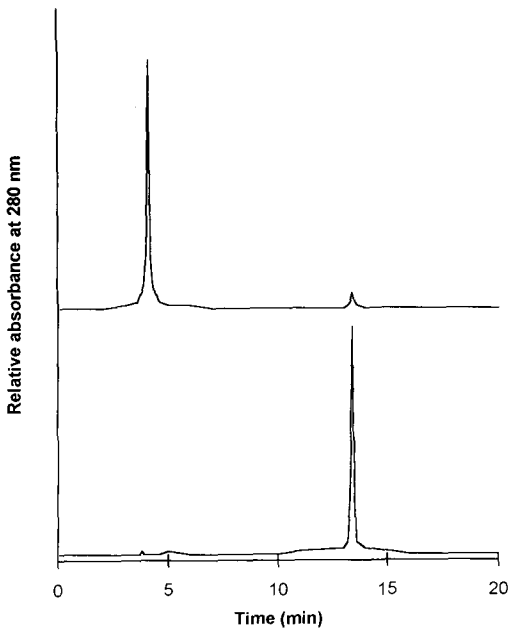

Figure 1. HPLC analyses of 2MBP and its hydrolysis products. The column was a Vydac Protein and Peptide C18, 4.6 × 250 mm. Solvent 1, 10 mM TFA in a 90/10 water/acetonitrile solution; solvent 2, 10 mM TFA in acetonitrile. The flow rate was 1 ml/min. The elution was performed as follows: 0–6 min, solvent 1; after 6 min, a gradient with 5%/min increasing solvent 2 was applied. *A* before hydrolysis; *B* after hydrolysis.

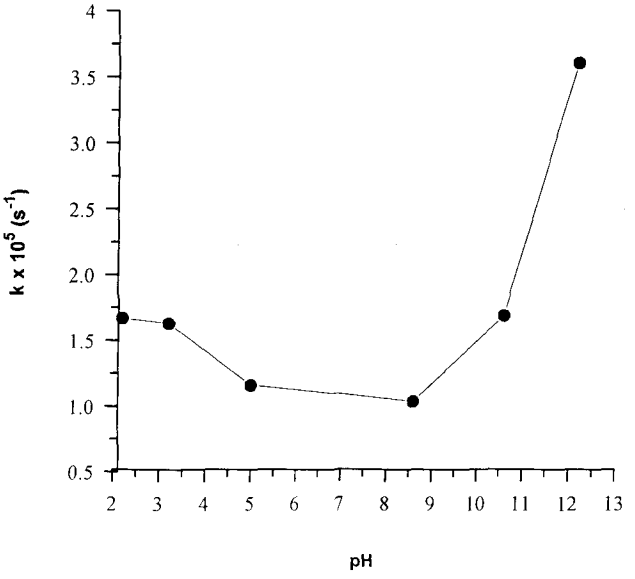

Figure 2. Spontaneous hydrolysis of 2MBP at different pH values. Solutions of 2MBP (4 mM) dissolved in the appropriate buffers at the indicated pH values were incubated at 25 °C. The hydrolysis was followed by measuring the residual substrate at varying time intervals. Ordinates: k indicates the apparent first orders kinetic constants for 2MBP hydrolysis.

Table. The main kinetic parameters of acylphosphatase isoenzymes.

	ACY-1	ACY-2	ACY-M
BP K_m (mM)	0.17 ± 0.01	0.10 ± 0.01	0.80 ± 0.05
BP V_{max} (U/mg)	9167 ± 242	8082 ± 201	4027 ± 133
2MBP K_m (mM)	0.18 ± 0.01	0.06 ± 0.01	0.73 ± 0.10
2MBP V_{max} (U/mg)	8323 ± 229	7200 ± 147	5278 ± 290
pH optimum (range)			
BP	4.8–5.8	4.8–5.8	4.8–5.8[a]
2MBP	4.8–5.8	4.8–5.8	4.8–6.4

ACY-1 and ACY-2 are organ common type isoenzymes, whereas ACY-M is the muscle type isoenzyme. [a]Taken from ref. 18.

its hydrolysis products. It can be seen that a major peak, eluted at 4.1 min, is present in figure 1A; this is accompanied by a small peak eluted at 13.4 min, the elution time of standard 2-methoxybenzoic acid. After hydrolysis, a single peak eluted at the 2-methoxybenzoic acid position is present (fig. 1B). The quantitative estimation of the two peaks eluted at 13.4 min indicates that only 4% free 2-methoxybenzoic acid is present in the purified product. The estimation of the free Pi content in the product was carried out by the method of Baginski et al.[24]. The result indicates that 4% Pi is present in the purified product. The measured molar ratio between 2-methoxybenzoate and Pi in the hydrolyzed product is 1.01. The estimation of the product purity by the Lipmann's method[23] indicates a 96% acylphosphate content. All the above findings demonstrate that the product is more than 95% pure.

The spontaneous hydrolysis rates of 2MBP solutions in the pH range 2.2–12.2 (at 25 °C) have been measured. Figure 2 shows the plot of the apparent first-order kinetic constants of 2MBP hydrolysis against pH. It can be seen that this acyl phosphate has maximum stability in the 5.0–8.6 pH range. At pH values higher than 8.6 the hydrolysis rate increases; this is probably caused by OH^- catalysis. On the other hand, at pH values lower than 5 the hydrolysis rate also increases. Thus, the maximum stability of this substrate is found in the region of the apparent pH optimum of acylphosphatase (see the table). At pH 5.3, the half-life of the product is 16.5 h.

Representative emission and excitation fluorescence spectra of 2MBP are shown in figure 3. These spectra have been recorded at room temperature in 0.05 M sodium acetate buffer, pH 5.3. It can be seen that two excitation bands (λ_{max} at 245 and 301 nm, fig. 3B) and one emission band (λ_{max} at 390 nm, fig. 3A) are visible upon excitation at 320 nm. Figure 3A shows also that the emission band disappears after hydrolysis. Degree of fluorescence correlates linearly with the concentration of the acyl phosphate in the 0.015–1.5 mM range (fig. 4A). At higher concentrations, a loss of linearity, probably due to inner filter effect, is seen. Figure 4B reports the fluorescence emission intensity at 390 nm measured in the 2–10 pH range. The graph shows that the increase in pH causes a transition in the fluorescence intensity that is probably due to an acidic group dissociation. A pKa value of 5.01 ± 0.05 SE has been calculated. Since the pKa for the dissociation of benzoyl phosphate monoanion to dianion is 4.8[26], the above

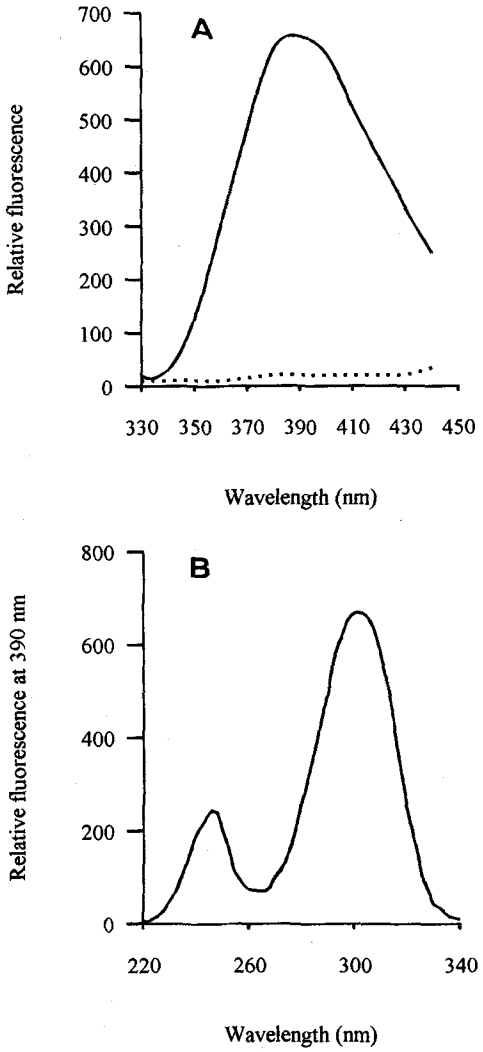

Figure 3. Fluorescence spectra of 2MBP and its hydrolysis products. The product (0.2 mM) was dissolved in 0.05 M sodium acetate buffer, pH 5.3. *A* emission spectra; the excitation wavelength was 320 nm. *B* excitation spectra. The continuous line refers to the unhydrolyzed product, whereas the dotted line refers to the completely hydrolyzed compound. The light path was 1 cm.

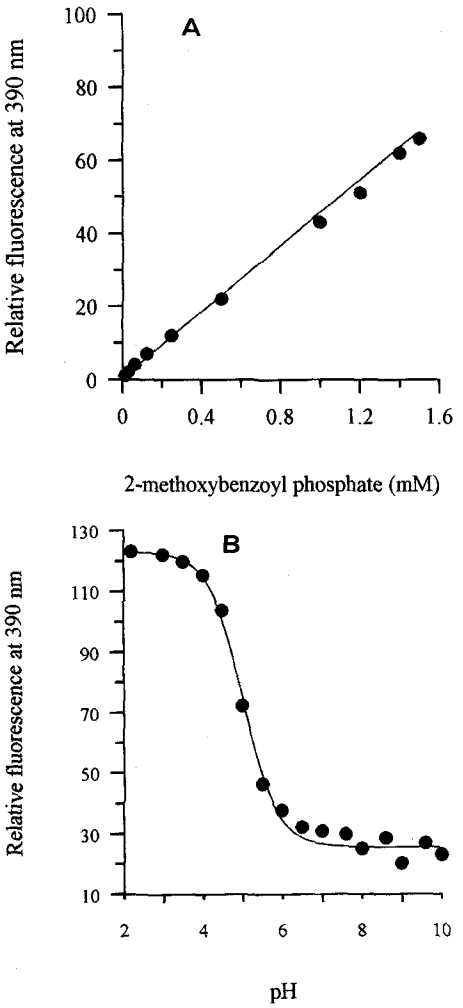

Figure 4. *A* Fluorescence emission intensities at different 2MBP concentrations. The product was dissolved in 0.05 M sodium acetate buffer, pH 5.3. *B* Fluorescence emission intensities of 2MBP at differing pH values. The product (0.2 mM) was dissolved in the following buffers: 0.05 M glycine-HCl, 2.2–3.0 pH range; 0.05 M sodium 3,3-dimethylglutarate, 3.5–7.6 pH range; 0.05 M Tris-HCl buffer, pH 8.0; 0.05 M glycine-NaOH, 8.6–10.0 pH range. Excitation wavelength was 340 nm. The light path was 1 cm.

results suggest that the pKa value of 5.01 refers to a similar dissociation of 2MBP monoanion to dianion (the structures of the two acyl phosphates are very similar). Thus the fluorescence intensities of 2MBP monoanion and 2MBP dianion differ from each other about four fold, the monoanion being a stronger fluorescence emitter than the dianion. Probably, the higher quantum yield of the monoanion with respect to the dianion is caused by the formation of a six-membered ring with the hydroxyl-hydrogen atom bonded to the carbonyl.

Figure 5 shows the UV absorption spectra of 2MBP and of its hydrolysis products at pH 5.3. It can be seen that hydrolysis, which produces 2-methoxybenzoic acid, causes a blue shift of the 300 nm absorption band displayed by 2MBP. This causes a strong reduction in the absorbance in the zone around 300 nm. The maxi-

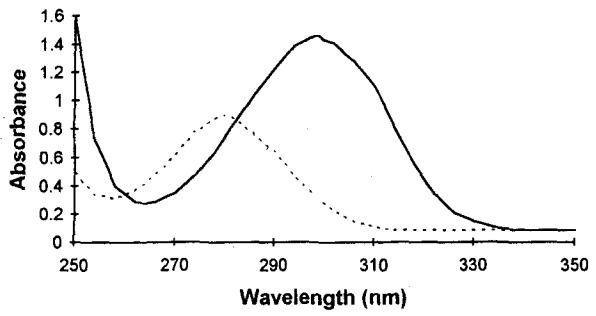

Figure 5. Near UV absorption spectra of 2MBP and of its hydrolysis products. The spectra were recorded on a Beckman Mod. 7500 spectrophotometer using 1 cm light-path cuvettes. The 2MBP concentration was 0.44 mM; the product was dissolved in 0.05 M sodium acetate buffer, pH 5.3. The continuous line refers to the unhydrolyzed product, whereas the dotted line refers to the completely hydrolyzed product.

mum $\Delta\varepsilon = 2720\ M^{-1}\ cm^{-1}$ has been found at 301 nm. This value is 4.3-fold higher than that displayed by BP at 283 nm (BP is the usually employed substrate for acylphosphatase assay[25]). Thus, the use of 2MBP instead of BP can improve the performance of a continuous UV-absorption test, since higher sensitivity is expected. In addition, we emphasize that the 300–320 nm wavelength range is useful to an acylphosphatase assay in the presence of most UV-absorbing metabolites and other compounds (fig. 5). Thus most of the potential aromatic inhibitors of the enzyme can readily be tested for their inhibition features.

The reliability of the fluorimetric and spectrophotometric acylphosphatase assay methods using 2MBP was checked. Both fluorimetric and spectrophotometric enzyme assays were performed in 1 cm light-path cuvettes (1 ml final volume) with 1.5 mM 2MBP dissolved in 0.1 M sodium acetate buffer, pH 5.3. Initial fluorescence or absorbance were recorded at 25 °C for 2–5 min to measure the spontaneous hydrolysis rate, and then acylphosphatase was added. The time-dependent decreases of fluorescence at 390 nm, or absorbance at 301 nm were followed. The reliability of the methods was checked by assaying the activity of solutions containing different amounts of acylphosphatase. Both continuous fluorimetric and spectrophotometric methods have been used. Figure 6 reports the result relative to ACY-M isoenzyme: the linear correlation between the differential fluorescence per min and the amount of enzyme added to the test mixture is shown. A good linear correlation between the absorbance changes per min at 301 nm and the enzyme added to the test mixture has also been found. The accuracy of both methods was checked by assaying 10 replicates of the same enzyme solution. We found 1.02 ± 0.04 µmol per ml of enzyme solution with the fluorescence test and 1.00 ± 0.03 µmol per ml of enzyme solution with the UV-spectrophotometric test. Assays with ACY-1 and ACY-2 isoenzymes produced comparable results.

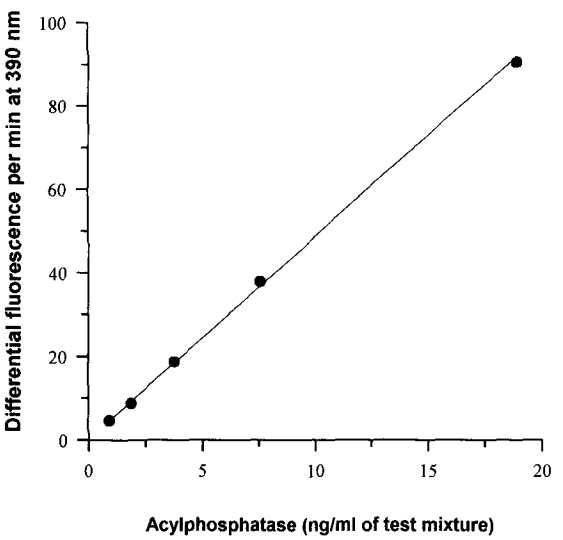

Figure 6. Correlation between acylphosphatase concentrations and fluorescence difference per min. The enzyme was assayed in 1 ml final volume of 1.5 mM 2MBP dissolved in 0.1 M sodium acetate buffer, pH 5.3, at 25 °C. The excitation was 340 nm.

The main kinetic properties of two isoenzyme types (common and muscle types) of acylphosphatases on 2MBP and on BP have been determined. The table reports the results relative to 2MBP, and these are compared with those of the same isoenzymes on BP. It can be seen that the K_m values relative to the organ common isoenzyme subtypes ACY-1 and ACY-2 on 2MBP are similar to those on BP. The V_{max} values of both subtypes on 2MBP are about 10% lower than those on BP. Furthermore, the K_m value relative to the muscle isoenzyme (ACY-M) is very similar to that on BP. On the other hand, the V_{max} value of ACY-M for 2MBP is about 30% higher than that for BP. All these findings suggest that 2MBP has properties that are very similar to those previously found for BP. This, together with its fluorescence and UV-absorption features, renders this compound a very useful substrate for routine and high sensitivity acylphosphatase assay. The table also reports the pH optimum range for the above isoenzymes using 2MBP and BP as substrates. It can be seen that all isoenzymes have a very similar pH optima.

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