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Studies on Alkaline Phosphatase

PHOSPHORYLATION OF CALF-INTESTINAL ALKALINE PHOSPHATASE BY ³²P-LABELLED PYROPHOSPHATE

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1. A purified preparation of alkaline phosphatase from calf-intestinal mucosa was phosphorylated by ^{32}P-labelled PP_i at a serine residue on the enzyme. Under the conditions employed, up to $0{\cdot}15\,\mu$M-labelled sites were obtained from $1\,\mu$M-[^{32}P]PP_i. 2. The phosphorylated enzyme was labile, the rate of dephosphorylation being similar to the overall rate of substrate hydrolysis. 3. A stopped-flow technique was used to determine the number of phosphomonoesterase active sites, which agreed with the number of ^{32}P-labelled sites. 4. It is concluded that calf-intestinal alkaline phosphatase is both a phosphomonoesterase and a pyrophosphatase.

Recently several groups of workers have claimed that alkaline phosphatase will hydrolyse PP_i and its monoesters (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1967; Fernley & Walker, 1967*b*). Other authors, notably Morton (1955), Portmann (1957), Stadtman (1961) and Dixon & Webb (1964), reported that with purified alkaline phosphatase this is not the case. The present investigation was carried out to examine this question further.

Under suitable conditions alkaline phosphatase can be specifically phosphorylated at a serine residue by P_i (Engström, 1961) or by phosphate esters (Engström, 1962; Fernley & Walker, 1967*a*). In the present paper evidence is given that [^{32}P]PP_i will also phosphorylate alkaline phosphatase and that the number of active sites so labelled agrees with the number of active sites determined by a stopped-flow technique with MUP.*

MATERIALS AND METHODS

Enzyme. Alkaline phosphatase from calf-intestinal mucosa [5mg. of protein/ml. of suspension in $(NH_4)_2SO_4$ solution] was kindly provided by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany (AP-1 15436 batch no. 6027510). Before use the preparation was dialysed at 2° against four changes of $0{\cdot}01\,I$ tris–acetic acid buffer, pH = pK of tris (8·75 at 2°), and stored at −20°.

Substrates. ^{32}P-labelled $Na_4P_2O_7$ (3mc) was purchased from The Radiochemical Centre, Amersham, Bucks. The specific activity on receipt was 76mc/m-mole. It was used without further purification and without added carrier. MUP was prepared as described by Fernley & Walker (1965).

Phosphorylation of alkaline phosphatase by [^{32}P]PP_i. Reactions were carried out in duplicate 16mm. × 90mm. round-bottomed borosilicate centrifuge tubes. To a solution (0·5ml.) containing 5–50 μg. of enzyme and 1mg. of bovine albumin (crystallized grade; Sigma Chemical Co., St Louis, Mo., U.S.A.) at 2° was added 0·5ml. of $2\,\mu$M-[^{32}P]PP_i in $0{\cdot}04\,I$ tris–acetic acid buffer, pH 4·3. After 5–180sec. the reaction mixture was treated with 0·2ml. of 20% (v/v) $HClO_4$ (Barman & Gutfreund, 1966). The protein precipitate was centrifuged, washed twice with 6ml. portions of 5% (v/v) $HClO_4$, dissolved in 90% (v/v) formic acid (0·1ml.) and reprecipitated with 6ml. of 5% $HClO_4$. After centrifuging there was negligible radioactivity in the supernatant. The protein pellet was dissolved in 1·0ml. of 90% formic acid and samples (50–100 μl.) were placed in aluminium planchets sprayed with Plastic-Film 348 (K. Frey, Wimmis, Switzerland) and carefully dried on a hotplate. Radioactivities were measured with a methane gas-flow counter (model FH407; Friesecke und Hoepfner G.m.b.H., Erlangen-Bruck, Germany). The efficiency of this counter was 69%.

Identification of phosphorylated products. A mixture of enzyme (0·3mg.), albumin (1mg.) and [^{32}P]PP_i ($1\,\mu$M) in 6ml. of $0{\cdot}02\,I$ tris–acetic acid buffer, pH 4·32, was incubated for 20sec. at 2° and then 1·2ml. of 20% $HClO_4$ was added to inactivate and precipitate the phosphoryl-phosphatase. The protein precipitate was washed free from non-bound [^{32}P]PP_i as described above. The final pellet was washed twice with acetone and then digested with 1ml. of 2N-HCl for 20hr. at 100° (Engström, 1961). The hydrolysate was freeze-dried and the residue dissolved in water (0·1ml.). Electrophoresis in N-acetic acid on Schleicher und Schuell chromatography paper (2043b Mgl) for 5hr. at 6v/cm. gave a clear separation of P_i and phosphoserine, the two ^{32}P-labelled products. Separation was also achieved by ascending paper chromatography, with the above paper and

* Abbreviation: MUP, 4-methylumbelliferyl phosphate monoester.

the following solvent systems: benzene–propionic acid–water (25:62:25, by vol.) (Ågren & Glomset, 1953); isobutyric acid–0·5N-NH_3 (5:3, v/v) (Magasanik, Vischner, Doniger, Elson & Chargaff, 1950). For quantitative counting, radioactive bands were located by radioautography (Kodak Kodirex X-ray film) and then eluted with 5ml. portions of water and 0·05N-HCl. Eluates were freeze-dried, taken up in 1ml. of water and duplicate 50μl. samples taken for counting as described above.

Time-course of hydrolysis of 1μM-[^{32}P]PP_i. Enzyme (25μg.), albumin (10mg.) and [^{32}P]PP_i (1μM) in 10ml. of 0·02I tris–acetic acid buffer, pH4·32, were incubated for 1–10min. at 2°. The reaction product [^{32}P]P_i was separated from [^{32}P]PP_i as follows.

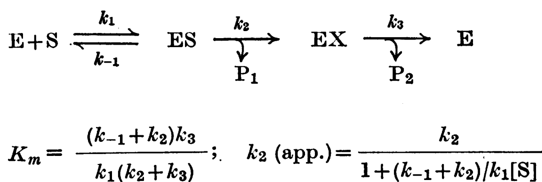
Samples (1·0ml.) were withdrawn into 0·35ml. of precipitating mixture (Sugino & Miyoshi, 1964): N-$HClO_4$, 4vol.; 10% (w/v) ammonium molybdate, 2vol.; 0·2M-triethylamine hydrochloride, 1vol. Carrier P_i (50μl. of 2mM-NaH_2PO_4) was added. The precipitate was centrifuged off and washed twice with 6ml. portions of washing mixture (prepared by adding a small amount of P_i to 1l. of solution containing 265ml. of precipitating mixture and then centrifuging. Acetone (0·2ml.) was added to the precipitate, with stirring, followed by 6ml. of the washing mixture. The precipitate was treated with acetone (1·0ml.) and centrifuged. Duplicate 50μl. samples of the clear supernatant were taken for counting as described above. The recovery of 1μM-[^{32}P]P_i was unaffected by the presence of 1μM-[^{32}P]PP_i.

Time-course of hydrolysis of 1μM-*MUP*. Enzyme (25μg.). albumin (10mg.) and MUP (1μM) in 10ml. of 0·02I tris–acetic acid buffer, pH4·32, were incubated for 1–10min. at 2°. Samples (1·0ml.) were withdrawn into 4ml. of M-K_2HPO_4–KOH buffer, pH10·4, containing 0·01M-EDTA. Fluorescence of 4-methylumbelliferone was measured in a Turner model 111 fluorimeter (G. K. Turner Associates, Palo Alto, Calif., U.S.A.) with 811 (primary) and 823 plus Kodak Wratten 2A (secondary) filters.

Stopped-flow technique. The stopped-flow apparatus of Gibson & Milnes (1964) was modified for fluorescence studies by substituting a 2mm.-bore fused-silica tube for the stainless-steel observation block, mounting the photomultiplier vertically and improving the optical system to exclude stray light. For determining the number of phosphomonoesterase active sites, equal volumes of enzyme (40μg./ml.) in 0·2% (w/v) albumin solution and MUP in 0·04I tris–acetic acid buffer, pH4·3, were rapidly mixed and the subsequent release of 4-methylumbelliferone was recorded on the screen of a Tektronix 564 storage oscilloscope. Fluorescence of 4-methylumbelliferone was excited with radiation from a d.c. stabilized mercury arc (model St41; Quarzlampen G.m.b.H., Hanau, Germany), the 313mμ line being isolated with a Jena UVIL interference filter. On the secondary side a Corning 3–73u.v. cut-off filter was used to remove scattered primary radiation and substrate fluorescence. The signal from the photomultiplier tube (model 6256A; Electrical and Musical Industries Ltd., Hayes, Middx.) was amplified through a high-speed picoammeter (model 417; Keithley Instruments Inc., Cleveland, Ohio, U.S.A.) and the picoammeter output was connected to the oscilloscope amplifier (type 2A63) through a 1MΩ resistor. The time-constant of the apparatus, minimum value 0·2msec., was controlled by varying the capacitance across the oscilloscope input.

RESULTS

Phosphorylation of alkaline phosphatase by [^{32}P]-PP_i. The conditions for possible phosphorylation were determined on the basis of earlier stopped-flow results with MUP (Fernley & Walker, 1967*a*). For a substantial amount of phosphoryl-enzyme to be present under steady-state conditions, it is necessary that the rate of phosphorylation (k_2) should be several times faster than the rate of dephosphorylation (k_3) in the reaction shown in Scheme 1. Here E and S are the free enzyme and substrate respectively, ES is the enzyme–substrate complex, EX is the phosphoryl-enzyme and P_1 and P_2 are the two reaction products. At pH4·4, with MUP as substrate, k_2 is 20–30-fold greater than k_3. If PP_i is also a substrate it should phosphorylate the enzyme under similar conditions and one should be able to isolate the phosphoryl-enzyme intermediate by acid inactivation.



Scheme 1.

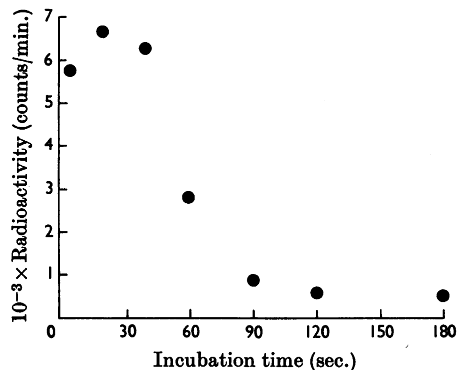


Fig. 1. Phosphorylation of calf-intestinal alkaline phosphatase (40μg. of protein in 1ml.) by 1μM-[^{32}P]PP_i. Mixtures were incubated at pH4·3 and 2° for the stated times and labelled enzyme was isolated as described in the Materials and Methods section. The radioactivities are the total counts/min. above background (25counts/min.) in each washed precipitate after subtracting the radioactivity in control mixtures containing 1mg. of albumin but no enzyme (mean value 530counts/min.). The standard, 1ml. of 1μM-[^{32}P]P_i, contained 41700counts/min.

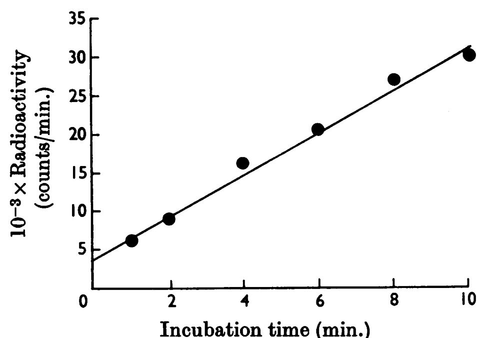


Fig. 2. Time-course of hydrolysis of 1μM-[^{32}P]PP_i by alkaline phosphatase ($2{\cdot}5\,\mu$g. of protein/ml.) at pH 4·3 and 2°. Radioactivities are expressed as counts/min. of [^{32}P]P_i/ml. of reaction mixture after subtracting the radioactivity in control mixtures containing substrate but no enzyme (mean value 3120 counts/min.). Separation of [^{32}P]P_i from [^{32}P]PP_i is described in the Materials and Methods section. The recovery of added [^{32}P]P_i ($1\,\mu$M) was 83% and the standard 1 ml. of 1μM-[^{32}P]P_i after recovery contained 22 360 counts/min. The line is derived statistically by the method of least squares (slope 2750 counts/min.^{2}, intercept 4020 counts/min.).

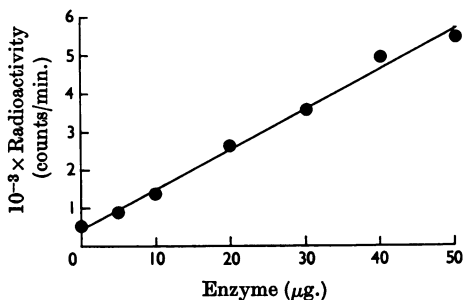


Fig. 3. Phosphorylation of alkaline phosphatase (5–50 μg. of protein in 1 ml.) by 1μM-[^{32}P]PP_i. Mixtures were incubated at pH 4·3 and 2° for 20 sec. and labelled enzyme was isolated as described in the Materials and Methods section. The standard 1 ml. of 1μM-[^{32}P]P_i contained 31 750 counts/min. For zero enzyme the counts refer to the 1 mg. of albumin present in each sample. The line is derived statistically (slope 106 counts/min./μg. of protein, intercept 432 counts/min.).

Phosphorylation of the enzyme preparation by [^{32}P]PP_i is shown in Fig. 1. The radioactivity is initially high and falls to a low value when the substrate has been hydrolysed. The time-course of hydrolysis of 1μM-[^{32}P]PP_i under the same conditions is shown in Fig. 2. The dependence of the amount of phosphoryl-enzyme isolated on the initial enzyme concentration is shown in Fig. 3.

To confirm that the phosphorylated enzyme was labelled with [^{32}P]P_i and not [^{32}P]PP_i a sample was hydrolysed with 2N-hydrochloric acid as described in the Materials and Methods section. Radioactive hydrolysis products were separated by paper electrophoresis and further characterized by subsequent paper chromatography. Only two radioactive bands were distinguished, running identically with P_i and phosphoserine. After elution from the paper electrophoretogram (with water and subsequently 0·05N-hydrochloric acid) the [^{32}P]P_i/[^{32}P]phosphoserine radioactivity ratio was 72:28 and the overall recovery of radioactivity was 84%. Under the same conditions of degradation phosphoserine was 75–80% hydrolysed. If the original label had been serine [^{32}P]pyrophosphate (which under the hydrolysis conditions might be expected to give [^{32}P]phosphoserine and [^{32}P]P_i), then the [^{32}P]P_i/[^{32}P]phosphoserine radioactivity ratio would have been about 6:1. The actual ratio found was 3:1, indicating that the original phosphoryl-enzyme contained all the ^{32}P label on a phosphoserine residue.

On this basis 1 mg. of protein contains 3·5 nmoles of labelled sites (Fig. 3). The rate of hydrolysis of PP_i under the same conditions as the labelling experiments is $0{\cdot}025\,\mu$mole/min./mg. of protein (Fig. 2). These data indicate that the turnover of labelled sites can be no more than 0·12 sec.$^{-1}$; a lower value is possible if there are more active sites or if there is an alternative pathway.

Phosphorylation of alkaline phosphatase by MUP. The reaction between $20\,\mu$g. of enzyme/ml. and $0{\cdot}55\,\mu$M-MUP is shown in Fig. 4(*a*). The trace can be fitted to the equation:

$$[\mathrm{P}]_t = at + b(1 - \mathrm{e}^{-ct})$$

where $[\mathrm{P}]_t$ is the concentration of the first reaction product, 4-methylumbelliferone, at time t, a is the steady-state rate, b ($\Delta[\mathrm{P}]_0$) is the difference between the extrapolated steady-state line and $[\mathrm{P}]_0$ the actual value of [P] at zero time, and c is a first-order constant describing the rate of decay of the reaction to the limiting steady-state rate. It may be determined by plotting $\log \Delta[\mathrm{P}]_t$ against t (Kezdy & Bender, 1962) as shown in Fig. 4(*b*). The slope of this plot is $-c/2{\cdot}303$. In Fig. 4, $a = 58{\cdot}5$ nM/sec., $b = 60$ nM and $c = 11{\cdot}7$ sec.$^{-1}$. By using the kinetic scheme appropriate to phosphoryl-enzyme formation (Scheme 1) the constants a, b and c can be formulated in terms of an active site concentration, $[\mathrm{E}]_0$ and two rate constants k_2(app.) and k_3:

$$a = \frac{[\mathrm{E}]_0 . k_2(\text{app.}) . k_3}{k_2(\text{app.}) + k_3}\,; \quad b = \frac{[\mathrm{E}]_0 . k_2(\text{app.})^2}{[k_2(\text{app.}) + k_3]^2}\,;$$
$$c = k_2(\text{app.}) + k_3$$

Substitution gives $[\mathrm{E}]_0 = 0{\cdot}070\,\mu$M, $k_2(\text{app.}) = 10{\cdot}8$ sec.$^{-1}$ and $k_3 = 0{\cdot}9$ sec.$^{-1}$. The concentration of

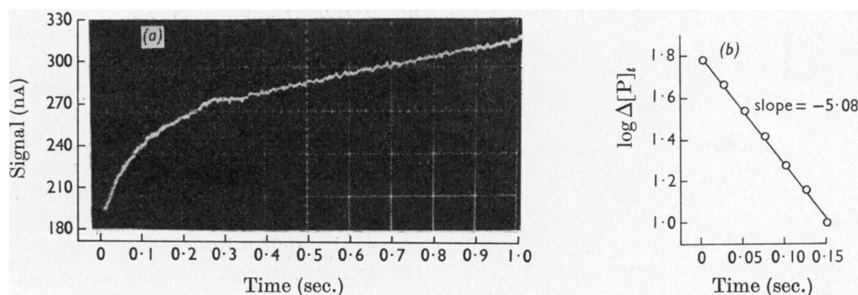


Fig. 4. (*a*) Reaction of alkaline phosphatase (20 μg. of protein/ml.) with MUP at pH 4·3 and 20°. The stopped-flow procedure is described in the Materials and Methods section. A signal of 10 nA corresponds to the release of 9·75 nM-methylumbelliferone. The time-constant of the apparatus was 2 msec. (*b*) Plot of log $\Delta[P]_t$ against time (sec.). The initial substrate concentration was calculated to be 0·55 μM and the initial 4-methylumbelliferone concentration 0·15 μM.

active sites therefore is 3·5 nmoles/mg. of protein and the amount of phosphoryl-enzyme under steady-state conditions is 92% of this value.

DISCUSSION

The use of ^{32}P-labelling for determining the concentration of active sites in alkaline phosphatase preparations is discussed by Barman & Gutfreund (1966), who point out the merits and some limitations of this method. The present results demonstrate that alkaline phosphatase can be phosphorylated by $[^{32}P]PP_i$ at a serine residue on the enzyme. The yield of phosphoryl-enzyme is substantial in that, on starting with 1 μM-$[^{32}P]PP_i$ and 50 μg./ml. of enzyme, 0·15 μM-labelled sites could be isolated after 20 sec. incubation (Fig. 3). Thus there is a rapid binding of 0·15 μM-substrate (Fig. 1) followed by hydrolysis of 0·41 μM-substrate (see Fig. 2), so that the substrate concentration remaining in solution at time of quenching is only 0·44 μM. The labelling could arise through hydrolysis of $[^{32}P]PP_i$ and a subsequent reaction of the enzyme with $[^{32}P]P_i$, but the data in Fig. 1 argue against such a mechanism. The maximum labelling attributable to $[^{32}P]P_i$, represented by the points at 120–180 sec. incubation where hydrolysis of the substrate is complete, amounts to only 8% of the total. The phosphoryl-enzyme must therefore be formed by a direct transfer of a phosphoryl group from $[^{32}P]PP_i$ to the enzyme. The lability of the phosphoryl-enzyme is also illustrated in Fig. 1 where, on exhaustion of the substrate, the concentration of labelled sites rapidly diminishes. On the basis that hydrolysis of PP_i proceeds entirely through a phosphoryl-enzyme intermediate, the half-life of the phosphoryl-enzyme is about 6 sec. The apparent half-life in Fig. 1 is about 10 sec. and this must be greater than the true half-life because phosphoryl-enzyme is being generated at all stages in the hydrolysis. Therefore the major pathway of PP_i hydrolysis must be via a phosphoryl-enzyme. The concentration of labelled sites agrees with the number of active sites determined with MUP (Fig. 4). Two results, the linearity of the time-course of PP_i hydrolysis and the maintained high level of phosphoryl-enzyme production in Fig. 1, suggest that K_m for PP_i is extremely low and consequently that the fractional labelling is correspondingly high.

Fernley & Walker (1967*b*) provisionally concluded that calf-intestinal alkaline phosphatase was both a phosphomonoesterase and a pyrophosphatase. The present results provide further evidence in support of that conclusion.

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