Biochimica et Biophysica Acta, 370 (1974) 477–486

BBA 67354

SUBUNIT STRUCTURE AND CATALYTIC ACTIVITY OF PIG KIDNEY ALKALINE PHOSPHATASE

INDRA RAMASAMY and PETER J BUTTERWORTH

Department of Biochemistry, Chelsea College, Manresa Rd, London SW3 6LX (U K)

(Received June 10th, 1974)

SUMMARY

1 Preparations of pig kidney alkaline phosphatase (EC 3 1 3 1) were shown to be homogeneous on polyacrylamide-gel electrophoresis

2 The apparent molecular weight of the enzyme determined by gel filtration was approx 185 000 After treatment with sodium dodecylsulphate, a single band with an apparent molecular weight of 80 000–90 000 was identified in polyacrylamide gels containing sodium dodecylsulphate Thus pig kidney alkaline phosphatase seems to be composed of two subunits

3 Chemical cross-linking with glutaraldehyde or dimethyl suberimidate prior to electrophoresis in polyacrylamide–sodium dodecylsulphate gels gave rise to two bands identified as free subunits and cross-linked dimers This evidence supports the suggestion that pig kidney alkaline phosphatase (like the enzymes from human placenta, calf intestine and *Escherichia coli*) consists of two very similar, probably identical, subunits

4 Titrations of the enzyme with $^{32}P_i$ at pH 5 0 (where phosphorylated enzyme is relatively stable) indicated that preparations were 45% pure, assuming that the subunits were identical and that two active sites were operational during the titration If only one active site per molecule becomes phosphorylated then the purity would be 90%

5 There was no evidence of negative cooperativity in the binding studies with P_i but extreme negative cooperativity leading to expression of only one site per dimer could not be excluded As the electrophoresis results seemed to indicate that the purity of the preparations was greater than 45%, there is some support for the belief that only one site was active

INTRODUCTION

Human placental alkaline phosphatase (EC 3 1 3 1) has been obtained in pure form [1, 2] and shown to be composed of two identical subunits with molecular weights of 62 000 [2] Similarly, calf intestinal enzyme has been shown to possess a dimeric structure, the subunits having a molecular weight of 69 000 [3] The existence of subunits in other mammalian alkaline phosphatases has been suggested when

interpreting results obtained in denaturation experiments with urea [4] and low pH [5, 6] In these studies positive identification of subunits was difficult because of the uncertain purity of the enzyme preparations and the impossibility of detecting alkaline phosphatase protein, present in very low concentration, once catalytic activity was lost Knowledge of the number of subunits present in a phosphatase molecule, and hence the possible number of active sites if the subunits are identical, is of interest because of the numerous reports that alkaline phosphatases of *Escherichia coli*, human placenta and calf intestine seem, under certain conditions, to show half-of-the-sites-reactivity [7–11]

Polyacrylamide-gel electrophoresis in the presence of sodium dodecylsulphate [12, 13] and chemical cross-linking of protein molecules [14–16] have become very useful techniques for studying oligomeric proteins This paper describes the application of such techniques to pig kidney alkaline phosphatase The enzyme of molecular weight 185 000 is shown to consist of two very similar subunits whose molecular weight is approx 80 000–90 000

EXPERIMENTAL

Enzyme

Alkaline phosphatase was purified from fresh pig kidney cortex as follows A portion of cortex tissue (3 2 kg) was minced at 4 °C then homogenised with 3 2 l of ice-cold water in a Waring Blendor Butanol (1 6 l) was added slowly to the homogenate with continuous stirring and then the mixture was allowed to stand overnight at 20 °C After straining through cheese-cloth, the extract was centrifuged at 18 000 × *g* for 40 min The aqueous layer was harvested and filtered through Hyflo-Super-Cel to remove small particles The pH of the filtrate was 6 5 Acetone at −20 °C was added to the filtrate at 4 °C until the final concentration of acetone was 60% The mixture was kept overnight at −20 °C then centrifuged at 15 000 × *g* for 30 min The precipitate, containing alkaline phosphatase, was suspended in 0 1 M Tris–HCl buffer (pH 7 6) and re-centrifuged at 15 000 × *g* to remove any undissolved residue (This step, and all subsequent ones, was carried out at 4 °C) The clear supernatant was brought to 55% satn with $(NH_4)_2SO_4$ Precipitated protein was discarded Alkaline phosphatase was precipitated at 75% satn with $(NH_4)_2SO_4$ The precipitate was dissolved in 0 1 M Tris–HCl buffer (pH 7 6) and dialysed for 16 h against the same buffer containing 1 mM $MgCl_2$

The dialysed enzyme was passed through a column (50 cm × 4 cm) of Sephadex G-200 equilibrated with the dialysis buffer The enzyme eluted as a single peak and was precipitated from the chromatographic fractions of highest specific activity by bringing to 75% satn with respect to $(NH_4)_2SO_4$ The precipitate was dialysed against 0 05 M Tris–HCl buffer (pH 7 6), containing 1 mM $MgCl_2$ for 16 h and then chromatographed in portions on DEAE-cellulose exactly as described previously [5] The enzyme was precipitated from the main peak of activity by 75% satn with $(NH_4)_2SO_4$ to effect concentration but this treatment caused a loss of up to 50% of the activity remaining at this stage

The procedure resulted in enzyme with a specific activity of 240–260 units/mg of protein but the overall yield of activity was less than 10% One unit of enzyme releases 1 μmole of *p*-nitrophenol/min from 3 mM *p*-nitrophenyl phosphate at 30 °C

and pH 10, 0 1 M $NaHCO_3$–Na_2CO_3 buffer The K_m of the enzyme is approx 0 3 mM under these conditions

Protein was determined from measurements of $A^{1\,cm}_{280\,mn}$ assuming that a solution containing 1 mg of alkaline phosphatase per ml had an extinction of 1 0 A close correlation was found in measurements of protein by absorbance at 280 nm and by the colorimetric procedure of Lowry et al [17] using bovine serum albumin as standard

Titration of alkaline phosphatase with [^{32}P]

The experiments were based on methods already described [18, 19] The reaction mixture of 0 25 ml volume contained carrier-free $^{32}P_i$ (25 μCi), varying amounts of unlabelled P_i (0–5 mM), 0 05 M acetate buffer (pH 5 0) and 22 units of enzyme (80 μg protein) Phosphorylation was carried out for 2 min at 0 °C and started by the addition of enzyme The incubation was terminated by spotting samples of 50 μl on discs (1 inch diameter) of Whatman 3 filter paper which were then immediately immersed in ice-cold 10% (w/v) trichloracetic acid Similar reaction mixtures but containing boiled enzyme or, alternatively, native enzyme plus 20 mM β-glycerophosphate served as controls The discs were washed 3 more times in 10% (w/v) trichloracetic acid at 0 °C followed by two washes in absolute ethanol, then one in diethyl ether [20] The papers were dried in air then transferred to 10 ml of scintillation fluid [21] and counted in a liquid scintillation spectrometer (Intertechnique ABAC SL40) Internal standards of ^{32}P were included to allow for the considerable amount of quenching

The effect of pH on the incorporation of $^{32}P_i$ into alkaline phosphatase was studied using a range of 0 05 M acetate and 0 05 M 2-amino-2-methyl-1-propanol–HCl buffers For buffers containing HCl, the molarity is that of the total base component after adjustment of the pH with HCl

Molecular weight determinations

The molecular weight of alkaline phosphatase was estimated by comparison of its elution volume with that of proteins of known molecular weight from a column (50 cm × 2 5 cm) of Biogel A 1 5m The gel medium was equilibrated in 0 1 M Tris–HCl buffer (pH 7 6) and proteins were eluted with this buffer at a flow rate of 10–20 ml per h Lactate and malate dehydrogenases, human γ-globulin, bovine thyroglobulin and catalase were detected in column effluents either by determination of enzymic activity [22] or from measurements of extinction at 280 nm The peaks of alkaline phosphatase were identified by determination of the rate of hydrolysis of *p*-nitrophenyl phosphate at pH 10 The effect of including 0 1 mM $MgCl_2$ in the elution buffer on the apparent molecular weight of alkaline phosphatase was also investigated

The apparent molecular weight of subunits of alkaline phosphatase was determined from measurements of the rate of migration of the enzyme and protein markers on polyacrylamide gels containing sodium dodecylsulphate The protein markers included bovine serum albumin (monomer and dimer), pepsin, yeast alcohol dehydrogenase, thyroglobulin, urease, myoglobin and cytochrome *c* Protein bands were stained with Coomassie Blue The procedures for gel and sample preparation and the staining method were those of Fairbanks et al [13] The identification of protein

bands derived from alkaline phosphatase was achieved by phosphorylation of 20 μg enzyme with $^{32}P_i$ (2 5 μCi) in a volume of 80 μl at pH 5 0 and 0 °C, as described above, prior to treatment with sodium dodecylsulphate and electrophoresis The gels were stained for protein and then cut into 2-mm portions which were placed in 10 ml of scintillation fluid for counting in a liquid-scintillation spectrometer As a control, electrophoresis was carried out on samples in which 0 1 M-β-glycerophosphate was present during the incubation with labelled $^{32}P_i$

Chemical cross-linking

The enzyme preparation was dialysed against 0 05 M potassium phosphate buffer (pH 7 0) containing 2 mM $MgCl_2$ for 16 h Portions (0 2 ml) of dialysed enzyme containing 10–14 μg of protein were incubated for 2 h at room temperature with 2–50 μl of 25% glutaraldehyde solution [16] The mixture was then heated at 100 °C for 1 min with β-mercaptoethanol plus sodium dodecylsulphate Both of these reagents were at a final concn of 1% After cooling to room temperature, a few μl of 15% (w/v) sucrose solution plus 5 μg of the marker dye Pyronin Y were added to the mixture which was then subjected to electrophoresis on polyacrylamide gel [12] The electrophoresis was run for approx 2 h at 8 mA/tube in gels containing 5% acrylamide Protein markers were subjected to electrophoresis under identical conditions to allow the approx molecular weight of cross-linked enzyme to be estimated

Dimethyl suberimidate was prepared from suberonitrile (1,6-dicyanohexane [14] Immediately before use, dimethyl suberimidate was dissolved in 0 05 M triethanolamine–HCl buffer (pH 8 5) and a sample of the solution added to 0 1 ml of enzyme (7 μg) to give 0 1–6 0 μg of suberimidate in a reaction mixture of 0 15 ml containing 5 mM $MgCl_2$ The mixture was kept at room temperature for 3 h before treatment with sodium dodecylsulphate and subsequent electrophoresis [14] In some experiments the concentration of suberimidate was increased to 6 mg per 0 15 ml during the cross-linking procedure in case the concentration was too low in the original experiments and also to check that the observed effects could be distinguished from cross-linking between molecules of alkaline phosphatase resulting in large oligomeric proteins

Electrophoresis of native alkaline phosphatase

The preparation of polyacrylamide gels and separation of proteins was carried out as described by Davis [23] The enzyme was detected on gels by incubating at pH 10 (0 1 M Na_2CO_3–$NaHCO_3$ buffer) with 1 mM α-naphthyl phosphate plus Fast Blue B salt (1 mg/ml) to couple with the released α-naphthol

Materials

Naphthalene, ethylene glycol (specially purified), 2-ethoxyethanol, and scintillation grade 2,5-diphenyloxazole and 1,4-di-2-(5-phenyloxazolylbenzene) were all obtained from Fisons Scientific Apparatus, Loughborough Leics The reagents for polyacrylamide-gel electrophoresis and *p*-nitrophenyl phosphate were obtained from British Drug Houses, Poole, Dorset The proteins used in molecular weight estimations came from the Sigma Chemical Co , St Louis, Mo U S A as did Fast Blue B salt and the aqueous solution of glutaraldehyde Ralph Emanuel, Wembley, Middx supplied 1,6-dicyanohexane and the Radiochemical Centre, Amersham, Bucks

supplied carrier-free $^{32}P_i$ Biogel A 1 5m, 100–200 mesh was obtained from Bio-Rad Laboratories Richmond, California, U S A All other reagents were of the best grades available commercially

RESULTS AND DISCUSSION

Fig 1 shows the pattern of enzyme and protein distribution obtained on electrophoresis of kidney alkaline phosphatase preparations in polyacrylamide gel The phosphatase activity was found to be coincident with the main protein band suggesting that the preparation was essentially homogeneous The diffuse appearance of the bands probably reflects microheterogeneity originating from variable amounts of enzyme-bound sialic acid (unpublished observations) in common with other mammalian alkaline phosphatases [24–26]

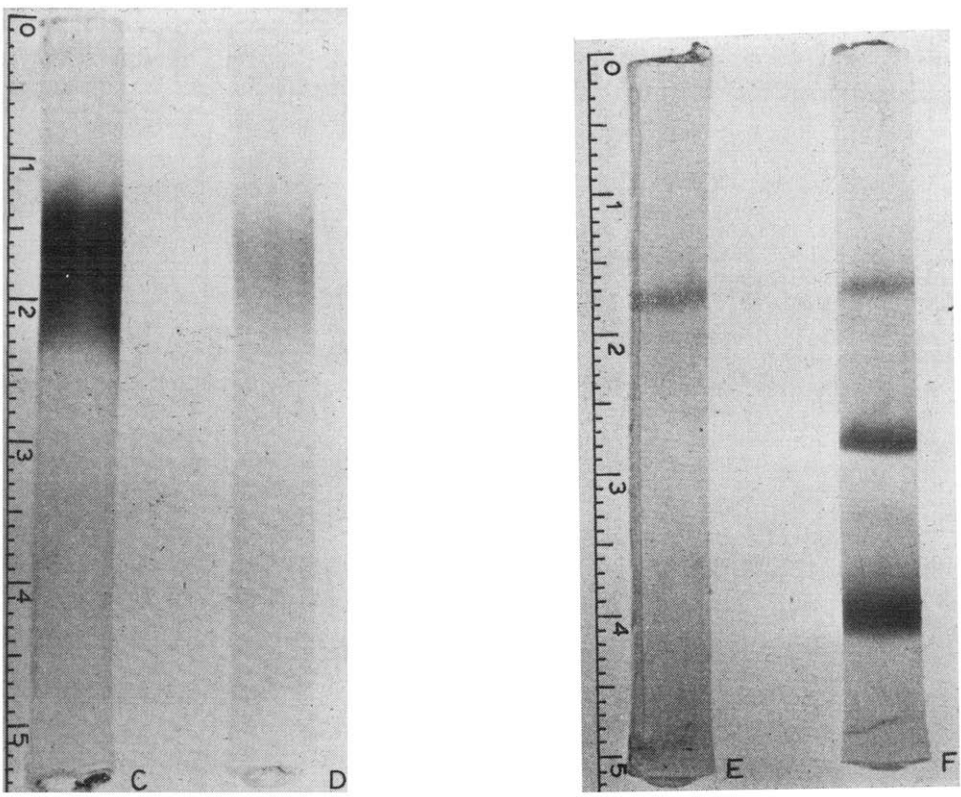

Fig 1 Polyacrylamide-gel electrophoresis of alkaline phosphatase The gels contained 7 5% polyacrylamide and separation was carried out at pH 8 0 [23] The point of sample application is at the top of the gels shown in the figure C, alkaline phosphatase located by incubation with α-naphthyl phosphate plus Fast Blue B salt, D, enzyme stained with coomassie blue

Fig 2 Polyacrylamide gel–sodium dodecyl sulphate electrophoresis The gels and samples were prepared according to Fairbanks et al [13] Electrophoresis was carried out at pH 7 6 in 5 6% polyacrylamide The point of sample application is at the top of the gels E, single band derived from alkaline phosphatase, F, bands derived from alkaline phosphatase (top), yeast alcohol dehydrogenase (middle) and cytochrome *c* (bottom) The proteins were stained with coomassie blue

After treatment with sodium dodecylsulphate only one protein band was usually detected in alkaline phosphatase preparations (Fig 2) but some preparations did contain trace amounts of a foreign protein that stained with Coomassie Blue The main band in these and other gels was identified as originating from alkaline phosphatase since this was the only radioactive band after incubation of the enzyme with $^{32}P_i$ prior to the treatment of the enzyme with detergent and electrophoresis The molecular weight of this component was estimated to be 80 000–90 000 by comparison with the rates of migration of proteins of known molecular weight The molecular weight of the native enzyme determined by gel filtration on Biogel A 1 5m was approx 185 000 and the presence of 0 1 mM $MgCl_2$ did not affect this value The values obtained from electrophoresis and gel filtration suggest that the native molecule contains two subunits

That the subunits of kidney alkaline phosphatase are identical was suggested by the results obtained from chemical cross-linking Considerab e reaction occurred in the presence of glutaraldehyde (Fig 3) but much less with suberimidate even when this reagent was present at relatively high concentration The results with both reagents, however, were compatible with the existence of only one kind of subunit

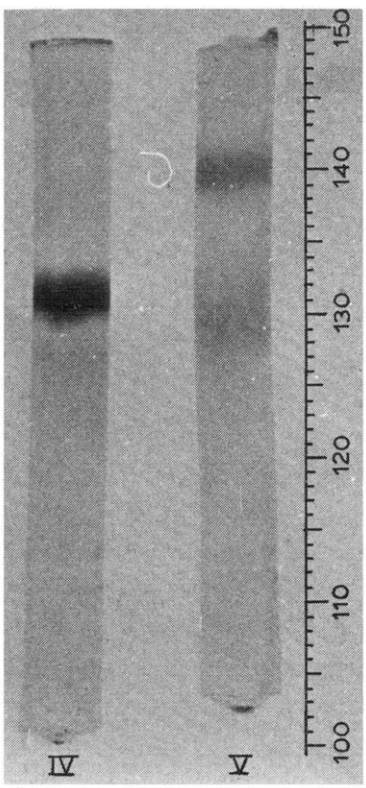

Fig 3 Electrophoresis of cross-linked alkaline phosphatase See Experimental for details of cross-linking with glutaraldehyde and sample preparation for electrophoresis The point of sample application is at the top of the gels IV, single band (monomer) derived from unreacted control, V, cross-linked dimer (top), free monomer (bottom)

since after dissociation in sodium dodecylsulphate, only two bands were detected on electrophoresis, one originating from cross-linked subunits, the other from free ones [14] The molecular weight of the band assumed to represent the dimer cross-linked with glutaraldehyde was approx 140 000 as judged by its rate of migration on sodium dodecylsulphate–polyacrylamide electrophoresis The dimer cross-linked with dimethyl suberimidate had a molecular weight of approx 160 000 It seemed unlikely that the small amounts of foreign protein present in some of the gels were involved in cross-linking with alkaline phosphatase when their intensity in stained gels was compared with that of the bands assumed to arise from phosphatase No proteins of large molecular weight representing intermolecularly linked enzyme were detected Also, no protein was detected at the top of the gels demonstrating the absence of material of such large size that gel penetration was prevented The small amount of reaction with suberimidate, a reagent that is claimed to react specifically with amino groups, probably means that the spacing between the reactive groups on suberimidate was not optimal for cross-linking alkaline phosphatase subunits No attempt was made to check this point however by using homologues of different chain length

For a reaction that may be written

$$E + P_0 \rightleftharpoons EP$$

Where E and EP represent the equilibrium concentrations of free enzyme active sites and phosphorylated enzyme respectively, provided that P_0 is in excess it can be shown that

$$\frac{P_0}{EP} = \frac{K}{E_0} + \frac{P_0}{E_0}$$

where E_0 and P_0 are total concentrations of the enzyme and orthophosphate respectively and K is the dissociation constant for the reaction Thus a plot of P_0/EP against P_0 should be linear with a slope of $1/E_0$ and an intercept on the horizontal axis at $-K$ [18, 19]

Fig 4 shows the results of phosphorylation experiments carried out on pig

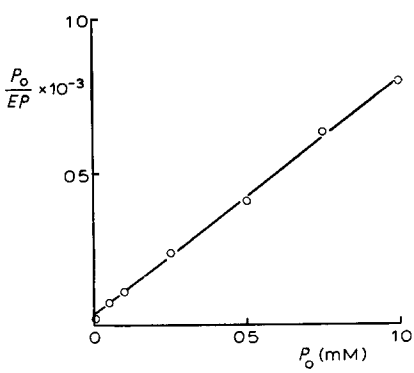

Fig 4 Titration of alkaline phosphatase with [^{32}P]orthophosphate at pH 5 0 Enzyme (80 μg) was incubated with various concentrations of P_i containing 25 μCi of $^{32}P_i$ at 0 °C Labelled protein was precipitated and counted as described in the Experimental Control incubations contained 20 mM β-glycerophosphate in addition to the other reagents

kidney alkaline phosphatase at pH 5 0 plotted as indicated above Over the range of phosphate concentration studied the plot was perfectly linear Assuming that all the phosphate binding sites are catalytic centres, the concentration of active sites determined from the plot was 1 6 μM and the value of K was approx 50 μM This value is likely to be imprecise because of the limited amount of data collected at P_0 levels lower than 50 μM From the concentration of active sites a catalytic centre activity of 855 s^- was calculated for kidney enzyme acting on p-nitrophenyl phosphate at pH 10 and 30 °C This figure compares with 2700 s^{-1} for bovine milk enzyme at 25 °C [18] 5030 s^- for human liver phosphatase and 6550 s^{-1} for human intestinal enzyme at 37 °C [19] and approx 500 s^- for both calf intestinal and human placental phosphatase [11, 27] Even though allowance must be made for the different conditions under which the measurements were made it is clear that alkaline phosphatases obtained from different animals and tissues show considerable variation in their catalytic activities

Assuming that pig kidney alkaline phosphatase contains two identical subunits and its molecular weight is 185 000, the purity of the best preparations was calculated to be approx 45% provided that active sites on both subunits are functional, i e phosphorylated at pH 5 0 If only one active centre is phosphorylated in the titration experiments then the purity would be greater than 90% Lacking firm evidence for half-of-the sites reactivity the high figure for the purity must be viewed with considerable caution at present although from the electrophoretic evidence the enzyme does seem to be better than 45% pure

The presence of 20 mM β-glycerophosphate inhibited the incorporation of $^{32}P_i$ into alkaline phosphatase and this finding was taken as proof that the labelling occurred at specific sites on the enzyme molecule Similarly, it was found that there was negligible incorporation into enzyme that had been inactivated by boiling or pre-treated with trichloracetic acid This evidence appeared also to show that phosphorylation did not occur during the washing process It is well known that alkaline phosphatases only form stable phosphoryl–enzyme intermediates at acid pH [28, 29] The effect of pH on the measured incorporation of label into pig kidney enzyme (Fig 5) is further indication that phosphate labelling occurs specifically Also that a

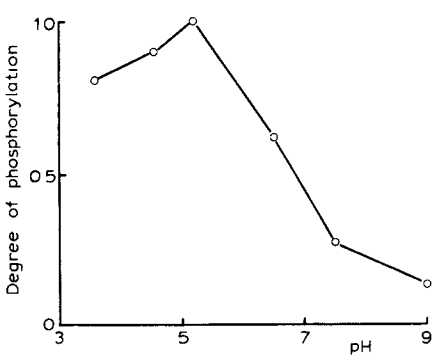

Fig 5 Effect of pH on the formation of stable phosphoryl–enzyme The phosphorylation procedure was as described in the legend to Fig 4 except that unlabelled P_i was maintained at 0 1 mM and incubations were carried out at a number of different pH values The degree of phosphorylation is expressed as a fraction of that occurring at pH 5 2

pH curve is obtained seems to provide evidence that bound phosphate is not lost during washing If loss did occur it is difficult to envisage how such a sharp optimum for incorporation would be seen Instead we would expect to find relatively small differences in the amounts of detectable incorporation at the various pH values tested

Linear plots of the kind shown in Fig 4 indicate only one type of binding site for phosphate but do not eliminate the possibility that only one site per dimer becomes phosphorylated Equilibrium dialysis has shown [30, 31] that *E coli* alkaline phosphatase possesses two sites which exhibit negative cooperativity [32] at alkaline pH Similar conclusions were reached for the phosphorylation of *E coli* enzyme at acid pH from labelling and stopped-flow kinetic studies [10, 31] and furthermore it seemed that activity may be limited to a single site at any one time, i e the enzyme exhibits half-of-the-sites reactivity [7] Human placental enzyme seems to share these properties since twice as many sites appear to be operating when a phosphorothioate ester is hydrolysed as during phosphate ester hydrolysis or during labelling of the enzyme with $^{32}P_i$ [33] If the kidney enzyme behaves like placental and calf intestinal enzymes then only a single site is phosphorylated in the experiments described above In a recent report, Bloch and Schlesinger [34] have shown by stopped-flow techniques how tightly bound P_i in enzyme preparations may account for some of the apparent negative cooperativity seen with *E coli* enzyme The presence of tightly bound P_i should not invalidate our estimates of the catalytic centre activity of pig kidney enzyme because of the large excess of exogenous P_i used in the phosphorylation experiments

Overall, these studies have shown that pig kidney alkaline phosphatase can be prepared in reasonably pure form and that the molecule contains two very similar, if not identical subunits No evidence was obtained for negative cooperativity between phosphate binding sites during titration with labelled orthophosphate, but negative cooperativity of an extreme kind leading to expression of only one site per dimer cannot be excluded by our results

REFERENCES

1 Ghosh, N K (1969) Ann N Y Acad Sci 166, 604–640
2 Harkness, D R (1968) Arch Biochem Biophys 126, 503–512
3 Fosset, M , Chappelet-Tordo, D and Lazdunski, M (1974) Biochemistry 13, 1783–1788
4 Butterworth, P J and Moss, D W (1967) Enzymologia 32, 269–277
5 Butterworth, P J (1968) Biochem J 108, 243–246
6 Scutt, P B and Moss, D W (1968) Enzymologia 35, 157–167
7 Levitski, A , Stallcup, W B and Koshland, D E (1971) Biochemistry 10, 3371–3378
8 Trentham, D R and Gutfreund, H (1968) Biochem J 106, 455–460
9 Halford, S E (1971) Biochem J 125, 319–327
10 Lazdunski, M , Petitclerc, C , Chappelet, D and Lazdunski, C (1971) Eur J Biochem 20, 124–139
11 Chappelet-Tordo, D , Fosset, M , Iwatsubo, M , Gache, C and Lazdunski, M (1974) Biochemistry 13, 1788–1795
12 Weber, K and Osborn, M (1969) J Biol Chem 244, 4406–4412
13 Fairbanks, G , Steck, T L and Wallach, D F H (1971) Biochemistry 10, 2606–2617
14 Davies, G E and Stark, G R (1970) Proc Natl Acad Sci U S 66, 651–656
15 Davies, G E and Kaplan, J G (1972) Can J Biochem 50 416–422
16 Griffith, I P (1972) Biochem J 126, 553–560
17 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–275

18 Barman, T E and Gutfreund, H (1966) Biochem J 101, 460–466
19 Moss, D W , Eaton, R H and Scutt, P B (1968) Biochim Biophys Acta 154, 613–614
20 Hucho, F , Randall, D D , Roche, T E , Burgett, M W , Pelley, J W and Reed, L J (1972) Arch Biochem Biophys 151, 328–340
21 Kobayashi, Y and Maudsley, D V (1969) in Methods of Biochemical Analysis (Glick, D , ed), Vol 17, pp 55–133, Interscience, New York, London, Sydney and Toronto
22 Andrews, P (1965) Biochem J 96, 595–606
23 Davis, B J (1964) Ann N Y Acad Sci 121, 404–427
24 Butterworth, P J and Moss, D W (1966) Nature 209, 805–806
25 Moss, D W , Eaton, R H , Smith, J K and Whitby, L G (1966) Biochem J 98, 32c–33c
26 Robinson, J C and Pierce, J E (1964) Nature 204, 472–473
27 Byers, D A , Fernley, H N and Walker, P G (1972) Eur J Biochem 29, 197–204
28 Fernley, H N (1971) in The Enzymes (Boyer, P D , ed), 3rd edn, Vol 4, pp 417–447, Academic Press, London and New York
29 Reid, T W and Wilson, I B (1971) in The Enzymes (Boyer, P D , ed), 3rd edn, Vol 4, pp 373–415, Academic Press, London and New York
30 Lazdunski, C , Petitclerc, C , Chappelet, D and Lazdunski, M (1969) Biochem Biophys Res Commun 37, 744–749
31 Simpson, R T and Vallee, B L (1970) Biochemistry 9, 953–958
32 Koshland, D E (1970) in The Enzymes (Boyer, P D , ed) 3rd edn, Vol 1, pp 341–396, Academic Press, London and New York
33 Fernley, H N (1973) Nat New Biol 241, 110–111
34 Bloch, W and Schlesinger, M J (1973) J Biol Chem 248, 5794–5805