The Purification of Fructose 1,6-Diphosphatase from Ox Liver and its Activation by Ethylenediaminetetra-acetate

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1. A procedure for the purification of ox liver fructose 1,6-diphosphatase is described. A number of criteria indicate that the enzyme was not subjected to any significant degree of proteolytic attack during the purification. 2. The molecular weight, amino acid composition and subunit molecular weight are reported. 3. The activation by EDTA was shown to be due to the chelation of heavy metals rather than by a more complex interaction with the enzyme as had previously been suggested.

The enzyme D-fructose 1,6-diphosphatase (EC 3.1.3.11) (fructose diphosphatase) catalyses the hydrolysis of D-fructose 1,6-diphosphate to D-fructose 6-phosphate plus P_i . The enzyme requires a bivalent metal ion, either Mg^{2+} or Mn^{2+} , for activity (Pontremoli *et al.*, 1968) and is allosterically inhibited by AMP (Taketa & Pogell, 1965; Underwood & Newsholme, 1965). The enzyme catalyses one of the irreversible steps of gluconeogenesis and it is now thought to be involved in the regulation of this pathway (see, e.g., Newsholme & Gevers, 1967).

Two types of mammalian liver fructose diphosphatase which differ markedly in some of their properties have been isolated and studied. The rabbit liver enzyme prepared by Pontremoli *et al.* (1965a) has been shown to have a pH optimum in the range pH 9.0–9.5 (Pontremoli *et al.*, 1965b). The purified enzyme has a molecular weight of 130 000 (Pontremoli *et al.*, 1965a) and contains two types of subunit, of molecular weights 29 000 and 36 000 (Sia *et al.*, 1969). This form of the enzyme contains no tryptophan (Fernando *et al.*, 1968) and has a K_i for AMP of about $100 \mu M$ (Pontremoli & Horecker, 1970). It has been termed 'alkaline' fructose diphosphatase (Traniello *et al.*, 1971). Subsequently, a form of the enzyme termed 'native' or 'neutral' fructose diphosphatase has been isolated from rabbit liver (Traniello *et al.*, 1971, 1972). This form has a pH optimum in the range pH 6.5–7.5 and a molecular weight of about 143 000. It is composed of four apparently identical subunits of mol.wt. 36 000, contains 4 mol of tryptophan per mol of enzyme and has a K_i for AMP of about $10 \mu M$. Similar forms of the enzyme have been isolated from ox liver (Byrne *et al.*, 1971) and rat liver (Traniello, 1974). It is known that treatment of purified 'native' fructose diphosphatase with proteolytic enzymes such as Pronase, subtilisin and papain, or with isolated liver lysosomes, can cause the

appearance of a species resembling 'alkaline' fructose diphosphatase (Geller *et al.*, 1971; Pontremoli *et al.*, 1971, 1973a,b; Traniello *et al.*, 1972). It has also been shown that 'alkaline' fructose diphosphatase is an artifact that can arise from 'native' enzyme during purification, presumably as a result of proteolytic attack on the enzyme (Nakashima & Horecker, 1971). It is therefore clear that any meaningful study of the regulatory properties of liver fructose diphosphatase must involve examination of the 'native' enzyme rather than the 'alkaline' enzyme.

One of the properties common to fructose diphosphatases from a variety of sources is that they are greatly stimulated by chelating agents when assayed at neutral pH (see, e.g., Rosen *et al.*, 1965; Tate, 1966; Van Tol *et al.*, 1972). The chelating agent that has been used to stimulate liver fructose diphosphatase most frequently is EDTA, but it can be replaced by other chelating agents, such as histidine (Geller *et al.*, 1971; Hers & Eggermont, 1964), and the fructose diphosphatase from *Candida utilis* can also be activated by 1,10-phenanthroline or 2,2'-bipyridyl (Rosen *et al.*, 1965). The degree of stimulation afforded by EDTA is much greater at pH 7 than at values above pH 9, where the enzyme is almost fully active in the absence of EDTA (Rosen *et al.*, 1965; Rosenberg *et al.*, 1973; Van Tol *et al.*, 1972). It is well known that fructose diphosphatases are very sensitive to inhibition by heavy-metal ions (Geller *et al.*, 1971; Rosen *et al.*, 1965; Underwood & Newsholme, 1965), but it has been claimed that the effect of EDTA is not simply a result of the chelation of such ions (Pogell *et al.*, 1968; Rosen *et al.*, 1965; Rosenberg *et al.*, 1973). However, the mechanism of the activation of fructose diphosphatase by EDTA and the pH-dependence of this process have not yet been satisfactorily explained. The existence of naturally occurring activators of fructose diphosphatase has been postulated by Pogell *et al.* (1968) and Allen & Blair (1972), who showed that the specific activity of crude rabbit liver fructose diphosphatase, in the absence of EDTA, rises

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with protein concentration. They interpreted this as suggesting the presence of a dissociable activator of fructose diphosphatase in the preparation. Some naturally occurring compounds, such as ATP, citrate and oleate, have been shown to activate purified fructose diphosphatase at neutral pH values in the absence of EDTA (Tashima *et al.*, 1971; Fu & Kemp, 1973; Baxter *et al.*, 1972), but none of these compounds have yet been shown to activate the enzyme in the presence of EDTA, and the mechanisms and relevance of these activation processes are as yet unclear.

In the present paper, a procedure for the purification of fructose diphosphatase from ox liver is described and the product is assessed to be 'native' enzyme by several criteria. The mechanism of activation of the enzyme by EDTA is also discussed.

Methods

Purification of fructose diphosphatase

Each step of the procedure was carried out as rapidly as possible at 0–4°C.

Step 1: extraction. Ox liver, obtained from freshly slaughtered animals, was cooled in ice and transported to the laboratory. Five 200g portions were homogenized in 4vol. of 0.15M-KCl for 15s at full speed in a Kenwood 'Chef' blender. The homogenate was strained to remove any large lumps of tissue and centrifuged at 12000g for 100min. After centrifugation, the supernatant was carefully decanted.

Step 2: pH treatment. The supernatant was adjusted to $\text{pH} 4.55 \pm 0.05$ by the dropwise addition of 5M-acetic acid, with stirring. The pH was immediately adjusted to 6.5 ± 0.1 by the dropwise addition of 5M-NaOH with stirring. The resulting suspension was centrifuged for 1×10^5 g-min and the supernatant was decanted.

Step 3: $(\text{NH}_4)_2\text{SO}_4$ fractionation. The supernatant was adjusted to 52% saturation (calculated by the method of Dixon, 1953) by the gradual addition of solid $(\text{NH}_4)_2\text{SO}_4$. The material was stirred for 30min and centrifuged for 1×10^5 g-min. The precipitate was discarded and the supernatant was adjusted to 62% saturation with $(\text{NH}_4)_2\text{SO}_4$, stirred and centrifuged as above. The precipitate was redissolved in 250ml of 5mm-malonic acid-NaOH buffer, pH 6.0 (buffer A). This material was desalted by passage through a column (bed vol. 2 litres) of Sephadex G-25 which had been equilibrated with buffer A.

Step 4: gradient elution from CM-cellulose. The desalted material was loaded on a column (5cm $\times$ 10cm) of CM-cellulose which had been equilibrated in buffer A. The column was washed with 1 litre of buffer A and developed with a 2-litre linear gradient of 5–30mm-malonic acid-NaOH buffer, pH 6.0. The flow rate was 400–500ml/h and 15ml fractions were

collected. Fructose diphosphatase activity was eluted in a single sharp peak in 18–20mm buffer. Fractions having fructose diphosphatase activity greater than 0.5 unit/ml were pooled and desalted into 5mm-malonic acid-NaOH buffer, pH 6.5 (buffer B), on a column (bed vol. 2 litres) of Sephadex G-25.

Step 5: substrate elution from CM-cellulose. The desalted material from step 4 was loaded on a column (2cm $\times$ 10cm) of CM-cellulose which had been equilibrated with buffer B. The column was washed with buffer B after loading until the effluent was protein-free, as judged by its absorbance at 280nm. The column was then developed with a 0.1mm solution of fructose diphosphate in buffer B adjusted to pH 6.5 with NaOH. The flow rate was 50–60ml/h and 5ml fractions were collected. Fructose diphosphatase activity was eluted in a sharp peak with the fructose diphosphate front. All fractions containing fructose diphosphatase at a specific activity of greater than 10 units/mg were combined and dialysed twice against 3 litres of 20mm-triethanolamine hydrochloride-KOH buffer, pH 7.5, containing 2mm-MgSO₄ and 1mm-EDTA (buffer C). After dialysis, the enzyme was concentrated by ultrafiltration to give a protein concentration of 4–6mg/ml.

Step 6: gel filtration on Sephadex G-200. The concentrated material was loaded on a column (3cm $\times$ 60cm) of Sephadex G-200 equilibrated with buffer C. The column was developed with buffer C at a flow rate of 10ml/h, and 3ml fractions were collected. Fractions containing fructose diphosphatase at a constant high specific activity were pooled and concentrated by ultrafiltration to give a protein concentration of 3–4mg/ml. For storage, $(\text{NH}_4)_2\text{SO}_4$ was added to 70% (w/v) saturation and the enzyme was kept as a suspension at 4°C.

By using this procedure, an overall purification of 2000–2500-fold was obtained as a routine, with the final specific activity of the enzyme and the overall yield being 21–25 units/mg and 18–25% respectively. The results of a typical purification are shown in Table 1 and the profile from a typical Sephadex G-200 column (step 6) is illustrated in Fig. 1. The 'pH ratio' (see below) and AMP sensitivity of the enzyme did not change throughout the purification and were constant across the peak of fructose diphosphatase eluted from the column.

Assay methods

Spectrophotometric assays were performed at 30°C in 1cm-path-length cuvettes in a Gilford 240 recording spectrophotometer. The reactions were initiated by the addition of the enzyme to be assayed to the remainder of the assay mixture. One unit of enzyme activity is defined as the amount needed to catalyse the disappearance of 1 μmol of substrate or the appearance of 1 μmol of product/min.

Table 1. Purification of fructose 1,6-diphosphatase from ox liver

Fraction	Vol. (ml)	Enzyme activity at pH7.2 (units/ml)	Protein concn. (mg/ml)	Specific activity at pH7.2 (units/mg)	'pH ratio'	Percentage inhibition given by 15 μ M-AMP	Purification (fold)	Yield (%)
Step 1	3600	1.1	108	0.01	2.10	54.3	1	100
Step 2	2550	1.2	34	0.04	2.05	55.9	4	79
Step 3	127	12.0	26	0.46	1.95	51.7	46	40
Step 4	330	4.1	1.8	2.3	2.03	53.6	230	35
Step 5	69	15.2	1.0	15.2	2.06	56.1	1520	27
Step 6	8.8	106	4.6	22.5	2.04	54.9	2250	25

Fig. 1. Gel filtration of fructose 1,6-diphosphatase on Sephadex G-200 in the final step of the purification procedure (step 6) Details of the gel-filtration procedure are given in the text. Each fraction was assayed for enzyme activity at pH7.2 in the presence and absence of 15 μ M-AMP and at pH9.5 by using the coupled assay described in the text. Δ , Protein concentration (% of the maximum value); $\bullet$, fructose diphosphatase activity at pH7.2 (% of the maximum value); $\square$, specific activity at pH7.2 (% of the maximum value); $\circ$, % inhibition given by 15 μ M-AMP at pH7.2; $\blacksquare$, 'pH ratio' as defined in the text.

As a routine fructose diphosphatase was assayed by using a coupled system in which the fructose 6-phosphate produced is used to reduce NADP⁺ in the presence of excess of glucose phosphate isomerase and glucose 6-phosphate dehydrogenase. Each cuvette contained either 50mM-triethanolamine hydrochloride-KOH buffer, pH7.4, or 50mM-glycine-KOH buffer, pH9.6, plus 100mM-KCl, 0.1mM-EDTA, 2.1mM-MgSO₄, 100 μ M-fructose diphosphate, 0.15mM-NADP⁺, 5 units of glucose phosphate isomerase, 3 units of glucose 6-phosphate dehydrogenase, and fructose diphosphatase in a final volume of 1.0ml. The final pH values in the cuvettes were 7.2 and 9.5 respectively, and the reduction of NADP⁺ was monitored at 340nm. In some experiments at pH9.5, the glycine-KOH buffer was replaced by 50mM-diethanolamine-HCl buffer to lessen the chelation of metal ions.

In some experiments, fructose diphosphatase activity was assayed by monitoring the release of P_i

from fructose diphosphate. Each assay mixture was incubated at 30°C and comprised, in a final volume of 10ml, 50mM-triethanolamine hydrochloride-KOH buffer, pH7.2, or 50mM-glycine-KOH buffer, pH9.5, plus 100mM-KCl, 0.1mM-EDTA, 2.1mM-MgSO₄ and 0.1mM-fructose diphosphate. Fructose diphosphatase was added at zero time, and at times 1, 3, 5 and 7 min, 2.5ml samples were withdrawn and analysed for P_i by the method of Michelsen (1957). In this procedure the reaction was stopped by lowering the pH of the medium to pH2. The components of the fructose diphosphatase assay did not interfere with the P_i determination, except that solutions of fructose diphosphate and AMP were found to be contaminated with about 3 and 2% P_i respectively. Assays were performed under conditions such that the liberation of P_i was linear with time up to 7 min. The activity of fructose diphosphatase measured by the coupled assay was always within 10% of its activity found by using the P_i-release assay under equivalent conditions.

Pyruvate kinase was assayed in a mixture comprising 50mM-triethanolamine hydrochloride-KOH buffer, pH7.2, 100mM-KCl, 20mM-MgSO₄, 1mM-phosphoenolpyruvate, 2mM-ADP, 0.2mM-NADH, 5 units of lactate dehydrogenase and enzyme. The oxidation of NADH was monitored at 340nm. β -Galactosidase was assayed in a mixture comprising 10mM-Tris-acetate buffer, pH7.5, 10mM-MgCl₂, 100mM-NaCl, 1.75mM-*o*-nitrophenyl galactoside and enzyme. The liberation of *o*-nitrophenol was monitored at 412nm. Lactate dehydrogenase was assayed in a mixture comprising 100mM-potassium phosphate buffer, pH7.0, 0.2mM-NADH, 1mM-pyruvate and enzyme. The oxidation of NADH was monitored at 340nm. Malate dehydrogenase was assayed in a mixture comprising 100mM-potassium phosphate, pH7.0, 1mM-oxaloacetate, 0.2mM-NADH and enzyme. The oxidation of NADH was monitored at 340nm. The protein concentrations of crude fractions were measured by the method of Kalckar (1947). The concentrations of samples of purified fructose diphosphatase were calculated from their absorbances at 280nm. A solution of fructose diphosphatase at 1mg dry wt. of protein/ml had an absorbance of 0.890 at 280nm.

Polyacrylamide-gel electrophoresis

Electrophoresis in 7% (w/v) polyacrylamide gels at pH8.9 was carried out as described by Davis (1964). Gels were stained for protein with Amido Black [1% (w/v) in 7% (v/v) acetic acid] and were destained by washing in 7% (v/v) acetic acid. Gels to be stained for fructose diphosphatase activity were washed once with 50mM-glycine-KOH buffer, pH10.0, containing 100mM-KCl, immediately after electrophoresis had ceased. They were then incubated in a staining 'cocktail' comprising 50mM-glycine-KOH buffer, pH10.0, 100mM-KCl, 5mM-fructose diphosphate, 8mM-MgSO₄ and 8mM-CaCl₂. After about 20min at 30°C, white bands of precipitated calcium phosphate were clearly visible and were photographed by reflected light against a dark background. Control gels, run without enzyme or incubated without fructose diphosphate, MgSO₄ or CaCl₂, gave no such bands. Electrophoresis in 10% (w/v) polyacrylamide gels with 2.7% cross-linkages, in the presence of 0.1% sodium dodecyl sulphate, was carried out as described by Bryce & Crichton (1971). Protein samples were denatured in 1% sodium dodecyl sulphate at 100°C as described by Pringle (1970). The subunit molecular weight of fructose diphosphatase was estimated from a calibration curve of log(subunit mol.wt.) plotted against electrophoretic mobility obtained with the marker proteins bovine serum albumin (mol.wt. 68000; Tanford *et al.*, 1967), catalase (subunit mol.wt. 60000; Sund *et al.*,

1967), carboxymethylated aldolase (mol.wt. 44000; I. Gibbons, personal communication), pepsin (mol.wt. 35000; Bovey & Yamari, 1960), trypsin (mol.wt. 23300; Weber & Osborn, 1969) and lysozyme (mol.wt. 14300; Weber & Osborn, 1969). Polyacrylamide gels in the presence of 8M-urea were run as described by Anderson *et al.* (1969).

Treatment of buffers with Chelex 100

This was performed by a modification of the method of Willard *et al.* (1969). Chelex 100(Na⁺ form; 100-200 mesh) was washed several times with glass-distilled water and a suspension of the resin was then titrated to the pH value of the buffer to be treated with either HCl or NaOH as appropriate. The buffer was mixed with 0.2 vol. of settled resin and the mixture was stirred at room temperature (about 20°C) for 30min. The resin was then removed by centrifugation, the Chelex-treated buffer was carefully decanted and its pH checked.

Ultrafiltration

This was carried out in Amicon ultrafiltration cells over Diaflo PM10 membranes at 4°C under a pressure of N₂ of 200kPa (about 30 lb/in²).

Amino acid analysis, peptide 'fingerprinting' and N-terminal determination

Fructose diphosphatase was reduced and carboxymethylated as described by Anderson *et al.* (1969). For amino acid analysis, 1mg of the carboxymethylated protein was hydrolysed with 5 drops of 5.7M-HCl *in vacuo* at 105°C for 22h and the residue after drying was analysed in a Beckman 120C automatic analyser, the precision of the results results obtained was estimated to be $\pm 5\%$. Tryptophan was determined separately by the method of Edelhoch (1967). Tryptic digestion and peptide 'fingerprinting' were carried out as described by Harris & Perham (1965). Paper electrophoresis was performed at pH6.5 and the solvent used for paper chromatography was acetic acid-butan-1-ol-pyridine-water (3:15:10:12, by vol.).

N-Terminal amino acid determinations were made with dansyl chloride (Gray & Hartley, 1963; Woods & Wang, 1967).

Gel-filtration experiments

Gel filtration was carried out on a column (2cm $\times$ 36cm) of Sephadex G-200 equilibrated in 20mM-triethanolamine hydrochloride-KOH buffer, pH7.4, containing 0.15M-KCl and 2mM-dithiothreitol. Molecular weights were estimated from a linear calibration

curve of elution volume against logarithm of molecular weight obtained with the marker proteins β -galactosidase (mol.wt. 520000; Ullmann *et al.*, 1968), pyruvate kinase (mol.wt. 237000; Steinmetz & Deal, 1966), lactate dehydrogenase (mol.wt. 140000; Castellino & Barker, 1968) and malate dehydrogenase (mol.wt. 65000; Andrews, 1965).

Analytical ultracentrifugation

This was carried out by using schlieren optics in a Spinco model E ultracentrifuge. The solvent used was a phosphate-NaCl buffer, pH 6.5 and 10.1, which contained 4.595 g of Na_2HPO_4 , 12 H_2O , 1.561 g of KH_2PO_4 and 2.923 g of NaCl/l. The sedimentation-velocity experiments were carried out at 52000 rev./min and the diffusion experiments at 4400 rev./min. All experiments were performed at $21 \pm 1^\circ\text{C}$.

Materials

Yeast glucose 6-phosphate dehydrogenase was prepared by a slight modification (Nimmo, 1974) of the method of Noltmann *et al.* (1961). Malate dehydrogenase, glucose phosphate isomerase, lactate dehydrogenase (pig muscle), β -galactosidase, catalase, pyruvate kinase, NADP⁺, fructose diphosphate (D-fructose 1,6-diphosphate), phosphoenolpyruvate (K^+ salt), oxaloacetic acid, pyruvate, triethanolamine hydrochloride and NADH were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Pepsin was purchased from Calbiochem Ltd., London W1H 1AS, U.K., and trypsin was obtained

from Miles-Seravac Ltd., Maidenhead, Berks., U.K. Chelex 100 was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. [$2\text{-}^{14}\text{C}$]EDTA (Na^+ salt, 19.8 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. CM-cellulose (CM52) was obtained from H. Reeve Angel Ltd., London EC4V 6AY, U.K., and Sephadex gels were purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Water was double-distilled, the second distillation being from an all-glass still. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., or Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., and were of the highest quality available.

Results

Characterization of the purified enzyme

The parameter 'pH ratio' is defined as the activity of a sample of fructose diphosphatase at pH 7.2 divided by its activity at pH 9.5. The pH optima of 'native' and 'alkaline' fructose diphosphatase differ, and this parameter should be a sensitive indicator of proteolytic attack on the enzyme. The 'pH ratio' and AMP sensitivity of fructose diphosphatase did not change throughout the purification (see Table 1), and in each step of the procedure involving column chromatography these parameters were constant across the fructose diphosphatase peak. This is illustrated for step 6 in Fig. 1. Further, the pH optima of the crude and purified enzymes, shown in Fig. 2, were very similar.

Fig. 2. pH optima of purified (a) and crude (b) fructose 1,6-diphosphatase

The enzyme activity was assayed by measuring the release of P_i as described in the text; 50 mM-imidazole-HCl buffer was used in the pH range 6.0–7.5, 50 mM-Tris-HCl buffer in the pH range 7.5–8.8 and 50 mM-glycine-KOH buffer in the pH range 9.0–10.0. The concentration of fructose diphosphate was 0.2 mM, that of MgSO_4 3.0 mM and that of EDTA 0.1 mM. These concentrations were known to be saturating at both pH 7.2 and 9.5. Also 100 mM-KCl was included in each assay, and the activity of the enzyme was relatively insensitive to ionic strength, rates changing by less than 10% in the range of KCl concentrations 50–200 mM.

Fig. 3. Dependence of sedimentation coefficient ($s_{20,w}$) on protein concentration for purified fructose 1,6-diphosphatase

Details of the method used in analytical ultracentrifugation are given in the text.

The purified enzyme appeared to be homogeneous by the criteria of polyacrylamide-gel electrophoresis and analytical ultracentrifugation. Plate 1 illustrates standard 7% polyacrylamide gels stained for protein or for fructose diphosphatase activity. In sedimentation-velocity measurements in the ultracentrifuge, the enzyme migrated as a single symmetrical peak, and the dependence of its sedimentation coefficient on protein concentration is shown in Fig. 3. A linear plot was obtained, as would be expected for a non-associating species, and the value of $s_{20,w}^0$ was estimated to be 7.64 ± 0.05 S. The diffusion coefficient, which is normally relatively unaffected by protein concentration (Tanford, 1961) was measured at 2.0 mg/ml and was estimated to be $0.51 \pm 0.01 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$. The partial specific volume, $\bar{v}$, of the enzyme was estimated from its amino acid composition, which is given in Table 2, by the method of Schachman (1957), and was found to be 0.739. Hence the molecular weight of the enzyme was estimated to be 140000 ± 3000 . To investigate the possibility that the molecular weight of the enzyme was altered during the purification, samples of crude fructose diphosphatase (after step 1 of the purification) and purified fructose diphosphatase were subjected to gel filtration on Sephadex G-200. The molecular weights of the crude and purified enzymes were estimated to be 141 000 and 143 000 respectively.

The purified enzyme gave only one band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate; the mobility of this band corresponded to a subunit molecular weight of 35500 ± 1500 . Carboxymethylated fructose diphosphatase was subjected to polyacrylamide-gel electro-

Table 2. Amino acid composition of fructose 1,6-diphosphatase

Carboxymethylated fructose 1,6-diphosphatase from ox liver was hydrolysed and analysed for amino acid content as described in the text. Tryptophan was determined, in a sample that had not been hydrolysed, by the method of Edelhoch (1967).

Amino acid	Content (residues per 140000 g of protein)
Lysine	102.5
Histidine	19.0
Arginine	46.6
Carboxymethylcysteine	22.7
Aspartic acid	134.1
Threonine	81.2
Serine	71.5
Glutamic acid	98.2
Proline	61.4
Glycine	103.2
Alanine	112.9
Valine	108.0
Methionine	33.5
Isoleucine	76.1
Leucine	105.5
Tyrosine	54.2
Phenylalanine	41.1
Tryptophan	4.4

phoresis in the presence of 8M-urea and again only a single protein band was observed. These results suggest that purified fructose diphosphatase contains only one type of subunit, and this conclusion was supported by two-dimensional 'mapping' of the peptides produced by a tryptic digest of carboxymethylated fructose diphosphatase. As shown in Fig. 4, 29–30 peptides were separated, with almost no 'core' material left at the origin. This value, compared with the 25–26 lysine and 11–12 arginine residues expected per unit of mol.wt. 35000 (see Table 2), suggests that the undissociated enzyme is a tetramer composed of identical subunits.

The amino acid composition of carboxymethylated fructose diphosphatase is given in Table 2. The presence of tryptophan was shown by spectrophotometric analysis (Edelhoch, 1967) and this result was confirmed qualitatively by examination of the fluorescence spectrum of the enzyme. This showed a strong emission peak at 340 nm, typical of tryptophan-containing proteins. Attempts to identify the *N*-terminal amino acid of carboxymethylated fructose diphosphatase were made by using the 'dansyl' technique. When 1 mg of protein was used, large spots corresponding to dansyl-ammonia, ϵ -dansyl-lysine and *O*-dansyltyrosine were observed, but no *N*-dansyl derivative could be detected. Although dansyl chloride does not react quantitatively with amino acids (Needle & Pollitt, 1965) and some losses

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of purified fructose 1,6-diphosphatase

Details of the electrophoretic and staining procedures are as given in the text; 7% polyacrylamide gels were used and each gel was loaded with 30 μ g of purified fructose 1,6-diphosphatase. Gel (a) was stained for protein and gel (b) for fructose 1,6-diphosphatase activity and photographed by reflected light against a dark background.

Fig. 4. Tryptic 'fingerprint' of purified fructose 1,6-diphosphatase

Carboxymethylated fructose 1,6-diphosphatase was digested with trypsin and the resulting peptides were separated by electrophoresis and chromatography as described in the text. The peptides indicated by broken lines stained only very faintly.

are expected during the procedure, the system of detection of the dansyl derivatives by their fluorescence can permit detection of quantities as low as 0.1 nmol (Crowshaw *et al.*, 1967). Since 1 mg of fructose diphosphatase corresponds to about 30 nmol of subunits, it seems that less than about 1% of the subunits can have a free *N*-terminal amino acid.

Activation of fructose diphosphatase by chelating agents

Fructose diphosphatase can be activated by a variety of chelating agents, and it was therefore important to investigate the chelating properties of the buffers used to assay the enzyme. This was done by following the titration of 8-hydroxyquinoline by Mg^{2+} ions (Burton, 1959). The triethanolamine-HCl and diethanolamine-HCl buffers were used, and there was no significant change in the response of the 8-hydroxyquinoline to Mg^{2+} ions on decreasing the concentration of either of the buffers by a factor of 50. This suggested that the buffers chelated negligible quantities of Mg^{2+} ions.

When fructose diphosphatase was assayed in 50 mM-triethanolamine hydrochloride-KOH buffer containing 100 mM-KCl at pH 7.2 in the absence of any

Table 3. Activation of fructose 1,6-diphosphatase by chelating agents

Fructose 1,6-diphosphatase was assayed at pH 7.2 by the coupled assay described in the text and in the presence of the agents indicated in the presence or absence of 10 μ M-EDTA. All assays were performed in the presence of a total concentration of 5.0 mM- Mg^{2+} . The results are expressed in terms of the activity at pH 7.2 in the presence of 10 μ M-EDTA but with no other additions. Abbreviation: EGTA, ethanedioxybis(ethylamine)tetra-acetate.

Agent	Concn.	Activity (%)	
		No EDTA	Plus 10 μ M-EDTA
None	—	8	100
ATP	1 mM	60	98
Citrate	1 mM	21	105
Citrate	3 mM	39	101
Histidine	1 mM	88	94
Imidazole	5 mM	74	91
EGTA	100 μ M	75	100
8-Hydroxyquinoline	20 μ M	48	87

chelating agents, it exhibited only a small proportion (about 8% at 5 mM- Mg^{2+}) of its activity in the presence of 10 μ M-EDTA. Several chelating species were capable of activating the enzyme in the absence of EDTA, and these are listed in Table 3. When the enzyme was assayed in 50 mM-diethanolamine-HCl buffer containing 100 mM-KCl at pH 9.5, however, it was almost completely active in the absence of EDTA, and the addition of EDTA to 1 mM (with the free Mg^{2+} ion concentration kept at 2.0 mM) resulted in a stimulation in activity of only 5%. When equal volumes of the two buffers were mixed, titrated either to pH 7.2 with HCl or to pH 9.5 with KOH, and used to assay fructose diphosphatase, the enzyme still required EDTA at pH 7.2 but not at pH 9.5. The pH-dependence of the EDTA requirement of fructose diphosphatase cannot therefore be the result of the presence of either an inhibitor only in the pH 7.2 buffer or an activator only in the pH 9.5 buffer.

The requirement of purified fructose diphosphatase for EDTA at pH 7.2 was investigated by assaying the enzyme in the presence of different concentrations of EDTA, by using either the coupled assay or the P_i -release assay. The enzyme required EDTA in both assay systems, and in quantitative terms the EDTA requirement was not significantly affected by changes in the concentrations of fructose diphosphate (10 μ M–1 mM), AMP (0–50 μ M) or KCl (0–150 mM) present in the system. However, the effects of EDTA on fructose diphosphatase at pH 7.2 depended on the nature and concentration of the activating cation. This is illustrated in Fig. 5, which shows that, as the concentration of free bivalent cation increased, both

Fig. 5. Activation of fructose 1,6-diphosphatase by EDTA at pH 7.2 in the presence of Mg^{2+} or Mn^{2+}

The activity of fructose diphosphatase was measured at pH 7.2 by using the coupled assay described in the text. The fructose diphosphate concentration used in these studies was 100 μM. (a) Assays in the presence of 0.2 mM (○) and 5.0 mM (□) Mg^{2+} . (b) Assays in the presence of 10 μM (□), 100 μM (○) and 1.0 mM (Δ) Mn^{2+} .

Fig. 6. Role of EDTA in the activation of fructose 1,6-diphosphatase

The activity of the enzyme was assayed at pH 7.2 and at a series of EDTA concentrations in the presence of 2 mM-free Mg^{2+} by using the coupled assay described in the text. □, Enzyme assayed in normal untreated buffer; ○, enzyme assayed in buffer that had been treated with Chelex 100; ●, enzyme desalted into buffer that had been treated with Chelex 100 by gel filtration on a column (1 cm × 30 cm) of Sephadex G-50 and was assayed in a mixture that had been treated with Chelex 100.

the rate observed in the absence of EDTA and the amount of EDTA required to give full activation of the enzyme increased. These effects were more marked when Mn^{2+} was used than with Mg^{2+} . This agrees with the results of Rosenberg *et al.* (1973) on 'native' rabbit kidney fructose diphosphatase, but in

direct contrast with the results of these authors, the dependence of rate on EDTA concentration was clearly not hyperbolic, but rather was similar to a titration curve, especially when Mg^{2+} was used. This suggests that EDTA may activate fructose diphosphatase by titrating out some inhibitor present in the assay systems, presumably a heavy-metal ion.

Effects of treatment of the assay buffers with Chelex 100

When 50 mM-triethanolamine hydrochloride-KOH buffer, pH 7.4, containing 100 mM-KCl, which had been treated with Chelex 100 ('Chelexed buffer'), was used instead of untreated buffer in the coupled assay system with Mg^{2+} , the activity of fructose diphosphatase observed in the absence of EDTA was increased but the EDTA requirement of the enzyme was not completely abolished. However, when fructose diphosphatase which had been stored in EDTA was desalted into 'Chelexed buffer' by gel filtration and assayed in a system in which all the components except Mg^{2+} had been treated with Chelex 100 ('Chelexed system'), it was maximally active in the absence of EDTA (Fig. 6). Corresponding experiments were carried out with Mn^{2+} instead of Mg^{2+} , and the results (Fig. 7) are qualitatively identical. Thus if all the components of the assay system (except the activating cation) are treated with Chelex 100, the requirement of fructose diphosphatase for EDTA at pH 7.2 can be eliminated.

The possibility that this result was due to the release of Na^+ from the chelating resin into the buffer, rather than the removal of inhibitory ions from it, was eliminated by the demonstration that NaCl, at a final concentration of 1 or 10 mM, had no effect on the EDTA requirement of fructose diphosphatase assayed in untreated buffer. It may also be argued that

Fig. 7. Abolition of the EDTA requirement of fructose 1,6-diphosphatase assayed in the presence of Mn^{2+}

The enzyme was transferred into buffer that had been treated with Chelex 100 and assayed at pH 7.2 in the presence of $100 \mu M$ -free Mn^{2+} as described in the legend of Fig. 6. □, Enzyme assayed in normal untreated buffer; ○, enzyme assayed in a mixture that had been treated with Chelex 100.

this result could have arisen from the release of a chelating species into solution during treatment of the buffers with Chelex 100. It is evident from Figs. 5(b) and 7 that this would have to be equivalent to 60 – $70 \mu M$ -EDTA in the buffer. In this case, however, the enzyme would have been fully active in the absence of EDTA when assayed with Mg^{2+} in 'Chelexed buffer' and the result represented in Fig. 6 shows that this was not observed. It seems therefore that Chelex 100 can activate fructose diphosphatase by removing inhibitory ions from the solution.

Lack of a direct interaction between fructose diphosphatase and EDTA

It has been suggested that EDTA, as the $MgEDTA$ complex, can interact directly with fructose diphosphatase (Rosenberg *et al.*, 1973). Although it seemed likely that EDTA activates fructose diphosphatase indirectly, as Chelex 100 appears to do, it was decided to test this theory by attempting to demonstrate a direct interaction between fructose diphosphatase and EDTA, by using the gel-filtration technique of Hummel & Dreyer (1962). Samples of purified fructose diphosphatase which had been freed of EDTA by gel filtration were passed through a column ($1 cm \times 30 cm$) of Sephadex G-50 equilibrated at $30^\circ C$ with $50 mM$ -triethanolamine hydrochloride-KOH buffer, containing $100 mM$ -KCl and $[^{14}C]$ -EDTA. At $10 \mu M$ -EDTA, no binding of EDTA to the enzyme could be detected, and this was not affected

by the presence of $2 mM$ - $MgSO_4$, $0.2 mM$ -fructose diphosphate or $0.2 mM$ -AMP. It was concluded that under these conditions the binding of EDTA to fructose diphosphatase amounted to less than $0.2 mol/mol$ of enzyme.

When fructose diphosphatase was assayed in the triethanolamine hydrochloride buffer used in these binding studies, it was greatly stimulated by $10 \mu M$ -EDTA (see Fig. 5). However, the enzyme concentration used in the binding studies was about three orders of magnitude greater than those used in the kinetic studies. The possibility that EDTA might bind to fructose diphosphatase only at low protein concentrations cannot be ruled out. It was found, however, that in fructose diphosphatase assays at pH 7.2 in the absence of EDTA, velocity was proportional to enzyme concentration over the studied range (0 – $15 \mu g/ml$), and this possibility therefore seems rather unlikely. Thus it appears that EDTA can activate fructose diphosphatase indirectly, without binding to the enzyme either as $MgEDTA$ (as suggested by Rosenberg *et al.*, 1973) or as free EDTA. This indicates that EDTA functions by chelating an inhibitor in solution rather than by interacting with an enzyme-inhibitor complex to form a ternary complex.

Inhibition of fructose diphosphatase by Zn^{2+}

The pH optimum of purified fructose diphosphatase which had been desalted into 'Chelexed buffer' by gel filtration was investigated by assaying the enzyme in buffers of different pH values which had been treated with Chelex 100 after addition of all the components of the coupled assay system except Mg^{2+} and fructose diphosphatase. The dependence of the sensitivity of fructose diphosphatase to inhibition by Zn^{2+} on pH was tested by including $1 \mu M$ - $ZnSO_4$ in a parallel assay at each pH value. The results of this experiment are shown in Fig. 8, and comparison of Figs. 2 and 8(a) shows that treatment of the assay systems with Chelex 100 does not greatly alter the pH optimum of fructose diphosphatase. It was also found that this treatment did not affect the sensitivity of the enzyme to AMP.

Fig. 8(b) shows that the inhibition of fructose diphosphatase by Zn^{2+} is very dependent on the pH of the assay and the chelating properties of the buffers used. In imidazole buffers little inhibition of the enzyme could be detected; this correlates well with the observation that imidazole can activate fructose diphosphatase in the absence of EDTA (Table 3). In the non-chelating triethanolamine hydrochloride and diethanolamine buffers, however, the pH-dependence of the inhibition of the enzyme by Zn^{2+} seems to resemble the titration curve of a group with a pK_a of about 8.4. The solubility product

Fig. 8. Effect of pH on the activity of fructose 1,6-diphosphatase and the inhibition of the enzyme by Zn^{2+} in a Chelex 100-treated system

The enzyme was transferred into buffer that had been treated with Chelex 100 as described in the legend to Fig. 6. The activity was assayed in the presence of $100\mu\text{M}$ -fructose diphosphate and 2mM - Mg^{2+} in buffers that had been treated with Chelex 100 after the addition of all the components of the coupled assay except Mg^{2+} and fructose 1,6-diphosphatase. (a) Buffers used were as follows: $\square$, 50mM -imidazole-HCl containing 100mM -KCl; $\circ$, 50mM -triethanolamine hydrochloride-KOH containing 100mM -KCl; $\triangle$, 50mM -diethanolamine-HCl containing 100mM -KCl. The buffers contained no EDTA. (b) Enzyme assayed in the presence of $1.0\mu\text{M}$ - ZnSO_4 , which was added to each buffer system after the treatment with Chelex 100. The results are expressed as a plot of % inhibition (given by the ZnSO_4) against pH. The buffers used were as defined in (a).

of $\text{Zn}(\text{OH})_2$ is about 16 (Gubeli & Sainte-Marie, 1967), so that $1\mu\text{M}$ - Zn^{2+} would be expected to precipitate as $\text{Zn}(\text{OH})_2$ between pH 9 and 10, whereas the pK_a values of fructose diphosphate seem to be below pH 7 (McGilvery, 1965). It therefore seems likely that the 'titration curve' observed in Fig. 8(b) corresponds to the ionization of a group (or groups) on the enzyme with a pK_a of about 8.4, and that this

results in a loss of the sensitivity of fructose diphosphatase to inhibition by Zn^{2+} at high pH values.

Discussion

Fructose diphosphatase has been purified to homogeneity from ox liver by a procedure which gave good reproducibility in terms of overall yield and final specific activity. The specific activity of the product at pH 7.2 and 30°C was 21–25 units/mg. This is similar to the value of 14.6 units/mg at 23°C reported for 'native' rabbit liver fructose diphosphatase (Traniello *et al.*, 1971, 1972), but it is considerably higher than the value of 7 units/mg at 37°C reported for the enzyme purified from ox liver by Byrne *et al.* (1971).

It has been mentioned above (see the introduction) that there are considerable differences between 'native' and 'alkaline' fructose diphosphatases in the parameters pH optimum, AMP sensitivity, molecular weight, subunit structure and tryptophan content. It has been shown that samples of liver fructose diphosphatase which contain no tryptophan, and have therefore presumably been subject to partial proteolytic attack, can still retain a neutral pH optimum (Benkovic *et al.*, 1974; Pontremoli *et al.*, 1973c). This implies that demonstration of a neutral pH optimum is not a criterion sufficient to establish a sample of fructose diphosphatase as 'native'; rather, it is necessary to investigate all the parameters listed above, insofar as is possible, in both crude and purified fructose diphosphatases.

It has been shown (see Table 1) that the 'pH ratio' and AMP sensitivity of ox liver fructose diphosphatase did not change throughout the purification procedure described here. Equally, the pH optima and molecular weights of the crude and purified fructose diphosphatases were not significantly different. The results of the sodium dodecyl sulphate-gel and urea-gel electrophoresis and tryptic 'fingerprint' show that only one type of subunit, of mol.wt. 35 500, was present in the purified enzyme, which must therefore be a tetramer composed of identical subunits. Spectrophotometric analysis showed that 4 mol of tryptophan was present/mol of enzyme. These criteria all suggest that in qualitative terms, the purified enzyme can be described as 'native' unproteolysed fructose diphosphatase.

Pontremoli *et al.* (1973b) have shown that 'native' rabbit liver fructose diphosphatase is resistant to digestion with aminopeptidase M, and the α -amino groups of the 'native' ox liver enzyme are apparently resistant to carbamoylation (Byrne *et al.*, 1971). These results suggest that the *N*-terminal residues of 'native' fructose diphosphatase may be blocked in some way. Although the *N*-terminal residues of the 'alkaline' rabbit liver fructose diphosphatase of

Pontremoli *et al.* (1965a) have not been determined, it is clear that the attack of subtilisin on 'native' rabbit liver fructose diphosphatase does result in the appearance of free α -amino groups (Pontremoli *et al.*, 1973b). Thus another difference between 'native' and 'alkaline' fructose diphosphatase may be a blockage of the α -amino groups of the former enzyme, but not the latter enzyme. If this is the case, a possible method of estimating the degree of proteolysis of any sample of fructose diphosphatase in quantitative terms would involve a quantitative determination of its α -amino groups. Thus the failure to detect any *N*-terminal amino acid in the purified fructose diphosphatase by the dansyl method suggests that it contains less than about 1% proteolysed enzyme.

Fructose diphosphatases from a variety of sources are very sensitive to inhibition by heavy-metal ions, but the assumption that EDTA and other chelating agents activate the enzyme by preventing such inhibition has frequently been challenged (see the introduction). The results presented here show that a wide variety of chelating agents can activate fructose diphosphatase at neutral pH values, but that such agents are not required at alkaline pH. It has also been shown that the requirement of the enzyme for a chelating agent at neutral pH can be abolished by the treatment of all possible components of the assay system with Chelex 100, and that [^{14}C]EDTA does not appear to interact with the enzyme. These results strongly suggest that EDTA does indeed activate fructose diphosphatase by chelating inhibitory metal ions. The major source of these ions is likely to be the glass-distilled water used for making up solutions, although the coupling enzymes used in the coupled assay may be a secondary source (see Fig. 6); the fact that EDTA is still required when fructose diphosphatase is assayed at pH 7.2 by the P_i -release assay shows that they cannot be the sole source of such ions.

It has been shown that the sensitivity of fructose diphosphatase to inhibition by Zn^{2+} appears to depend on the state of ionization of a group (or groups) in the enzyme with a pK_a of about 8.4. The 'low-pH' form of the enzyme seems very sensitive to this inhibition, whereas the 'high-pH' form is relatively insensitive to it. This probably explains why the enzyme does not appear to require EDTA at high pH values. There is known to be a radical change in the kinetic properties of the enzyme between pH 7.2 and 9.5 (H. G. Nimmo & K. F. Tipton, unpublished work), and it is interesting to speculate that the same ionization may be responsible for this also. It is not yet known whether the ionizing group is involved in the inhibition by Zn^{2+} directly or indirectly, and its nature is also unknown.

Several naturally occurring compounds are known to be capable of activating fructose diphosphatase in the absence of EDTA, although not in its presence.

The nature of many of these compounds, such as ATP (Tashima *et al.*, 1971), histidine (Hers & Eggermont, 1964), citrate and creatine phosphate (Fu & Kemp, 1973), suggest that they too may act as chelating agents, and the fact that they do not activate the enzyme in the presence of EDTA supports this. However, the activation by fatty acids (Baxter *et al.*, 1972; Carlson *et al.*, 1973) and phospholipids (Allen & Blair, 1972) is rather more puzzling.

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