Purification and characterization of the imidazoleglycerol-phosphate dehydratase of *Saccharomyces cerevisiae* from recombinant *Escherichia coli*

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The *HIS3⁺* gene of *Saccharomyces cerevisiae* was overexpressed in *Escherichia coli* and the recombinant imidazoleglycerol-phosphate dehydratase (IGPD) purified to homogeneity. Laser-desorption and electrospray m.s. indicated a molecular ion within 2 units of that expected (23833.3) on the basis of the protein sequence, with about half of the polypeptide lacking the N-terminal formylmethionine residue. IGPD initially purified as an apoprotein was catalytically inactive and mainly a trimer of *M_r* 70000. Addition of Mn²⁺ (but not Mg²⁺) caused this to assemble to an active (40 units/mg) enzyme (Mn-IGPD) comprising of 24 subunits (*M_r* 573000) and containing 1.35 ± 0.1 Mn atoms/polypeptide subunit. An enzyme with an identical activity and metal content was also obtained when the fermenter growth medium of recombinant *Escherichia coli* was supplemented with MnCl₂, and IGPD was purified through as Mn-IGPD rather than as the apoenzyme and assembled *in vitro*. Inhibition by EDTA indicated that the intrinsic Mn²⁺ was essential for activity. The retention of activity over time after dilution to very low concentrations of enzyme (< 20 nM) indicated that the metal remained in tight association with the protein. A novel continuous

assay method was developed to facilitate the kinetic characterization of Mn-IGPD. At pH 7.0, the *K_m* for IGP was 0.10 ± 0.02 mM and the *K_i* value for inhibition by 1,2,4-triazole, 0.12 ± 0.02 mM. In contrast with other reports, thiols had no influence on catalytic activity. The activity of Mn-IGPD varied with enzyme concentration in such a way as to suggest that it dissociates to a less active form at very low concentrations. Significant inhibition by the product, imidazole acetol phosphate, was inferred from the shape of the progress curve. Titration with, the potent competitive inhibitor, 2-hydroxy-3-(1,2,4-triazol-1-yl)propyl phosphonate indicated that Mn-IGPD contained 0.9 ± 0.1 catalytic sites/protomer. The activity nearly doubled in the presence of high concentrations of Mn²⁺; the apparent *K_s* for stimulation was 20 µM. The basis of this effect was obscure, since there was no corresponding increase in the titre of active sites. Neither was there a discernable shift in the values of *K_m* or *K_i* (above), although exogenous Mn²⁺ did reduce the optimum pH for *k_{cat}* from 7.2 to 6.8. On the basis of a single site/subunit, the maximum rate of catalytic turnover at 30 °C was 32 s⁻¹.

INTRODUCTION

Imidazoleglycerol-phosphate dehydratase (IGPD; EC 4.2.1.19) catalyses the dehydration (Scheme 1) of imidazoleglycerol phosphate (IGP) to imidazoleacetol phosphate (IAP) and is the seventh step in the pathway for the biosynthesis of histidine (Ames and Mitchell, 1955; Ames 1957). Genetic and biochemical data define ten enzyme-catalysed steps in histidine biosynthesis (Artz and Holzschu, 1983) which, in bacteria, are encoded by

nine genes. Histidine biosynthesis starts with the transfer of C-1 of phosphoribosyl pyrophosphate to N-1 of the purine ring of ATP. Later steps involve amido transfer from glutamine in the formation of the imidazole ring and amino transfer from glutamate to, ultimately, supply the amino group of histidine. In bacteria, the dehydratase activity can either be monofunctional [e.g. as in *Azospirillum brasilense* (Fani et al., 1989)] or combined with histidinol phosphate phosphatase (the ninth step in the pathway) in a single bifunctional enzyme encoded by the *hisB*

Scheme 1 Reaction catalysed by IGPD

Abbreviations used: IGPD, imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19); IGP, imidazoleglycerol phosphate; IAP, imidazoleacetol phosphate; PTH, phenylthiohydantoin; IAPT, imidazoleacetol-phosphate transaminase; IPTG, isopropyl β-D-thiogalactoside; GDH, glutamate dehydrogenase.

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gene [e.g. in *Salmonella typhimurium* (Loper, 1961) and in *Escherichia coli* (Chiariotti et al., 1986)]. These two activities of the enzyme from *S. typhimurium* have been separated *in vitro* by limited proteolysis (Staples and Houston, 1979). The yeast enzyme encoded by the *HIS3* gene (Fink, 1964) is monofunctional and only expresses dehydratase activity. It has been cloned, sequenced and functionally expressed in *E. coli* (Struhl et al., 1980).

The mechanism of dehydration is unknown and, with no imine or carbonyl group α to the departing proton, must be unusual (cf. Gerlt and Gassman, 1992). The apparent strict requirement for Mn^{2+} (Ames, 1957) might seem to argue for the involvement of this metal. Most feasible mechanisms would generate the enol of IAP as the initial product. The final ketonization of any such enol would also need to be an enzyme-catalysed step (Moore et al., 1993). Hawkes et al. (1993) described a novel class of herbicides, the triazole phosphonates, which act as potent inhibitors of IGPD. On the basis of the relationship between inhibition, activity as a substrate and chemical structure, it was proposed that catalysis involves a diazafulvene intermediate in which one of the phosphate oxygen atoms of IGP acts as an internal base to remove the proton from C-2 and, furthermore, that triazole phosphonates were effective inhibitors because of their close resemblance to this proposed intermediate. Amitrole (3-amino-1,2,4-triazole) is another herbicide known to inhibit IGPD (Hilton et al., 1965), although, in this case, inhibition is much weaker and its action on carotenoid biosynthesis is undoubtedly more significant (Burns et al., 1971).

IGPD is an enzyme which has not previously been characterized in great detail. It was partly purified from *Neurospora crassa* (Ames, 1957) and from *S. cerevisiae* (Wiater et al., 1971) to yield preparations with Mn^{2+} -dependent activities of 0.2–0.4 μmol of IAP formed/min per mg. Optimal activity also required a very high concentration of β -mercaptoethanol. The pure yeast enzyme was reported (Glaser and Houston, 1974) to be a protein of M_r 290 000 consisting of identical subunits of M_r 23 850 (Struhl, 1985) with a K_m of 0.54 mM and a specific activity of about 0.3 $\mu\text{mol}/\text{min}$ per mg. A recent paper (Mano et al., 1993) described the purification of a monofunctional IGPD activity from wheat. Again a reducing agent (mercaptoethanol) was found necessary for activity and, of a range of bivalent metal cations tried, only Mn^{2+} stimulated activity. At 30 °C, the wheat enzyme had a specific activity of 5.7 $\mu\text{mol}/\text{min}$ per mg, a K_m for IGP of 0.36 mM and a pH optimum near 6.6. Here we describe the expression of the *HIS3⁺* gene of *Saccharomyces cerevisiae* in *E. coli* and the first purification and characterization of IGPD (EC 4.2.1.19) in a substantially active form. We also describe a sensitive and continuous assay suitable for the accurate determination of K_m and K_i values.

MATERIALS AND METHODS

Chemicals and enzymes

Chemicals and biochemicals were of the highest grade available from BDH or Sigma. $(\text{NH}_4)_2\text{SO}_4$ was Aristar grade from BDH. Poly(ethylene glycol) 3350 was from Sigma. Other protein purification materials were from Pharmacia, Chelex 100 resin was from Bio-Rad and 2-hydroxy-3-(1,2,4-triazol-1-yl)propylphosphonate was synthesized as described by Cox (1983).

D-erythro-(2R,3S)-IGP was synthesized as described by Ames (1957). The final material was mainly erythro (*anti*), but also contained about 15% of the threo (*syn*) stereoisomer. The stereoisomers were conveniently distinguished by n.m.r. on the basis of slight differences in the chemical shifts of their imidazole protons. The enzyme-catalysed reaction (pure Mn-IGPD added

to 50 mM IGP adjusted to p^2H 7.5 containing 0.2 M KCl in 95% 2H_2O) was readily monitored by ^{13}C and 1H n.m.r. In agreement with the results obtained using wheat enzyme (Saika et al., 1993) the threo material was not a substrate, whereas the erythro diastereomer was completely transformed to IAP. Thus the erythro component was entirely the D enantiomer. N.M.r. was carried out using a JEOL GX 400 MHz n.m.r. instrument.

Binding of Mn^{2+} to IGP

The formation constant for the complex of Mn^{2+} with IGP was determined as described by Townsend (1954) by monitoring the broadening of the six-line e.p.r. spectrum of $Mn(\text{H}_2\text{O})_6^{2+}$. $MnCl_2$ (0.2 mM in 0.2 M triethanolamine buffer, pH 7.5) was titrated with increasing concentrations of IGP, and solution-state e.p.r. measurements were carried out in a standard flat cell at 20 °C and recorded at 9 GHz on a Varian E9 spectrometer.

M.s.

Protein samples at 1 mg/ml were prepared by extensive dialysis in Milli-Q water and then further diluted to about 10 pmol/ μl in a 1:1 mixture of water and acetonitrile containing 1% formic acid. Electrospray m.s. was carried out using a VG Bio-Q Triple Quad electrospray mass spectrometer in single quad mode. Samples were infused at 5 $\mu\text{l}/\text{min}$. Data were analysed and transformed to an M_r scale using the 'maximum entropy' method of analysis to deconvolute spectra (Ferrige et al., 1992). Matrix-assisted laser desorption time-of-flight m.s. was carried out using a Finnegan LaserMat. The matrix used was sinnapinic acid, and the calibration was external against carbonic anhydrase (M_r 29022.7).

N-terminal protein sequencing

A 10 μl portion of the same solution used for m.s. was analysed using an Applied Biosystems model 475 protein sequencer with on-line detection of phenylthiohydantoin (PTH) amino acids using a model 120A PTH analyser. Additional N-terminal sequencing of redissolved protein crystals was performed using an ABI model 476A protein sequencer. Protein crystals precipitated from solution in $(\text{NH}_4)_2\text{SO}_4$ (details from D. W. R. on request) were washed with stabilizing solution and then dialysed overnight against buffers not including precipitant. These samples were then run on an SDS/9%-polyacrylamide gel which was then electroblotted using the ProBlott system. Protein bands from the blot were applied to a Bioprene-treated glass-fibre disc and subjected to 11 cycles of Edman degradation. At each cycle, the released anilinothiozolinone-amino acid was converted into a more stable PTH derivative and identified by on-line h.p.l.c. using a reverse-phase C_{18} column.

PAGE of proteins

Samples were prepared in SDS buffer and run on 12% slab gels, prepared and run essentially by the method of Laemmli (1970).

Cloning and DNA-sequencing procedures

Plasmid DNA was isolated and manipulated by standard procedures (Sambrook et al., 1989). DNA fragments were recovered from preparative agarose gels (Seakem Genetic Technology Grade agarose from FMC Bioproducts) using the GENE-CLEAN Kit (Bio101 Inc.). DNA was sequenced on both strands by the dideoxynucleotide-termination method (Sanger et al., 1977) using the Sequenase Version 2 kit (U.S. Biochemicals

Corp.). Template DNA was prepared by alkaline denaturation of plasmid DNA (Chen and Seeburg, 1985) and synthetic oligonucleotide primers, based on the published sequence of the yeast *HIS3*⁺ gene (Struhl, 1985), primed the sequencing reaction at intervals of approx. 250 bp along the template.

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer using standard procedures. Following deprotection, the oligonucleotides were dialysed extensively against water. Concentrations were determined from A_{260} using a calculated absorption coefficient for each primer (Sproat and Gait, 1984). Sequencing and PCR primers were used without further purification.

For assembly of the part-synthetic IGPD gene, the full-length oligonucleotides were separated by preparative electrophoresis on a denaturing polyacrylamide gel and revealed by u.v. shadowing (Applied Biosystems, 1987). Oligonucleotide 1 and 2 (see below) were eluted into water and dialysed extensively. A 100 pmol portion of each was 5'-phosphorylated using T4 polynucleotide kinase. A 20 pmol portion of each phosphorylated oligonucleotide was annealed by heating to 80 °C and cooling to 22 °C over 3 h in a 40 μ l reaction volume containing 50 mM Tris/HCl buffer (pH 7.6)/10 mM MgCl₂.

Heterologous expression of the *S. cerevisiae* *HIS3* gene in *E. coli*

The plasmid pUC8Sc2676 which contained the *PET56-HIS3-DED1* region from the *S. cerevisiae* gene was generously given by Dr. Kevin Struhl of Harvard Medical School, Boston, MA, U.S.A. (Struhl, 1985). The *HIS3* gene was re-sequenced prior to expression-plasmid construction using a series of oligonucleotide primers based on the published sequence (Struhl, 1985). The native *HIS3* gene lacked a convenient restriction site close to the translation-initiation codon, the nearest unique site being *Hinf*I at position 571 [numbered as described by Struhl (1985) and deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number X03245]. We therefore replaced the promoter proximal region of the gene with a short synthetic DNA fragment to introduce a unique *Sca*I restriction site coincident with the second codon (Thr) in the yeast gene. The codon usage in this region was optimized for expression in *E. coli*. The pair of complementary oligonucleotides designed to introduce these alterations were oligonucleotide 1:

5'-AATTCAGTACTGAACAGAAAGCTCTGGTTAAACGT-ATCACCAACGAAACCAAG

and oligonucleotide 2:

5'-AATCTTGGTTTCGTTGGTGATACGTTTAACCAGAG-CTTCTGTTCACTAGT

The double-stranded DNA fragment generated by annealing these oligonucleotides had *Eco*R1 and *Hinf*I cohesive ends. The part-synthetic IGPD gene was assembled by ligating pUC18 (restricted with *Eco*R1 and *Bam*H1) with the 5' phosphorylated and annealed oligonucleotides (0.1 pmol) plus a 1250 bp *Hinf*I-*Bam*H1 restriction fragment isolated from pUC8Sc2676 harbouring the distal segment of the IGPD gene. The reconstructed region of the IGPD gene in the resulting plasmid, pIGPDMod1, was confirmed by DNA sequencing. Restriction of pIGPDMod1 with *Sca*I would have generated a blunt-ended DNA fragment immediately before, and in-frame with, the Thr codon.

The expression vector, pCH34, was provided by Dr. Mike Edge of Zeneca Pharmaceuticals. This plasmid is derived from

Figure 1 Schematic diagram (not to scale) of the plasmid pIGPDX34

Abbreviations: Apr, β -lactamase gene; cl-857, temperature-sensitive repressor gene; oLpL, strong leftward operator/promoter; rbs, ribosome-binding site; trpA term, transcription terminator from *trpA* gene; ori, origin of replication.

pAT153 and employs the strong leftward operator and promoter of bacteriophage lambda plus a two-cistron ribosome-binding site (identical with that in pCZ114; Schoner et al., 1984) for efficient translation of the transcribed mRNA. The plasmid also carries the *cl*⁸⁵⁷ gene encoding a temperature-sensitive repressor of the lambda promoter. Expression of heterologous genes cloned into the vector is induced by shifting the growth temperature from 37 °C to 42 °C.

The expression plasmid, pIGPDX34, was assembled by ligation of a 900 bp *Sca*I-*Xba*I fragment isolated from pIGPDMod1 into pCH34 previously restricted with *Kpn*I, blunt-end-repaired with T4 DNA polymerase, recut with *Sal*I, dephosphorylated and gel-purified. The blunt-end ligation results in precise in-frame fusion of the Thr codon to an ATG codon derived from the vector. *Xba*I and *Sal*I produce compatible cohesive ends, allowing ligation. The DNA sequence across the ribosome-binding site, initiation ATG codon and into the IGPD gene was confirmed. pIGPDX34 (Figure 1) was designed for expression of the native yeast enzyme in *E. coli* (i.e. without any amino-acid-sequence alterations as a result of the DNA manipulations).

E. coli MM294 harbouring the plasmid pIGPDX34 accumulated a polypeptide of M_r about 24000 on induction and growth at 42 °C. Yeast IGPD was expressed as a soluble and active enzyme. Scanning densitometry of Coomassie Blue-stained gels indicated that it was about 10% of the total cell protein after induction at 42 °C for 2 h (cf. Figure 2).

Homologous expression of the *E. coli* *hisC* gene in *E. coli*

The *hisC* gene encoding imidazoleacetol-phosphate transaminase (IAPT) was amplified from *E. coli* K12 chromosomal DNA by PCR. Primers were based on the known sequence of the *hisC* gene from *E. coli* (Grisolia et al., 1985). The plasmid pIAPT99 contained the entire coding region of the *hisC* gene plus the natural ribosome-binding site cloned downstream of the *trc* promoter in pTrc99A (Pharmacia). On induction with 0.1 mM

Figure 2 SDS/PAGE of IGPD

Gels contained 12% polyacrylamide and stained for protein with Coomassie Blue. Lanes a, b and c were fractions I, II and III of apo-IGPD obtained after gel filtration through Superdex 200 (cf. Table 1 and Figure 3). Lane d was Mn-IGPD assembled *in vivo* and purified from cells induced with additional Mn^{2+} . Lane e was Mn-IGPD isolated from fraction III of apo-IGPD after assembly with Mn^{2+} and rechromatography through Superdex 200 (cf. Figure 3e). Lane f was a whole extract of *E. coli* MM294 (pIGPDX34) uninduced, and lane g was the same 2 h after induction at 42 °C. Lane h contained Bio-Rad M_r markers, hen's-egg-white lysozyme (14 400), soybean trypsin inhibitor (21 500), bovine carbonic anhydrase (31 000), hen's-egg-white ovalbumin (45 000), BSA (66 200) and rabbit muscle phosphorylase *b* (97 400).

isopropyl β -D-thiogalactoside (IPTG), *E. coli* DH5 α harbouring pIAPT $\times$ 99 produced large amounts of the expected 39 300- M_r polypeptide in a soluble and enzymically active form.

Large-scale growth and induction of recombinant *E. coli*

MM294 (pIGPDX34) were grown to a high biomass at 37 °C and under constant aeration in a 12-litre fermenter using a nitrogen-rich low-casein medium (LCM50; Kara and Hockney, 1991). Cells were induced while they were still in exponential-phase growth [attenuance (*D*) about 20 at 550 nm] by raising the temperature to 42 °C for 1 h. After a further 1 h at 37 °C, the cells were cooled and harvested by low-speed centrifugation. The wet cell paste was then quickly frozen by dropping spatula-sized lumps into liquid nitrogen and stored at -80 °C until needed. The rate of growth decreased markedly immediately after induction. In a variation of the procedure aimed at assembling Mn-IGPD *in vivo*, the usual trace elements of Co, Ni and Zn were omitted from the medium. This appeared to have no adverse effect on growth. In addition, the medium was supplemented with 0.4 mM $MnCl_2$ 1 h before induction. With this pre-addition of Mn there was much less of a reduction in growth rate after induction (although a similar amount of IGPD was induced as in the experiment without additional Mn^{2+}). The normal yield of cells was 300–500 g wet weight from 12 litres of medium. *E. coli* DH5 α (pIAPT $\times$ 99) was similarly grown, but, in this case, induced by addition of 0.1 mM IPTG rather than an increase in temperature. Cells were harvested 4 h after induction in similar yield to that described above.

Determination of protein concentration

Protein was routinely determined by the method of Bradford (1976), with BSA as standard. Absolute concentrations of IGPD were determined on the basis of the colorimetric reaction of the four cysteine residues (at positions 81, 96, 156 and 178) with 5,5'-dithiobis-(2-nitrobenzoic acid) essentially as described by Means

and Feeney (1971). IGPD was first exchanged into thiol-free buffer by gel filtration and denatured with SDS. Estimates of IGPD concentration using the Bradford (1976) method averaged 109% of the value based on the content of cysteine thiol, and about 115% when measured by using the Biuret method (Gornall et al., 1949). Concentrations of IGPD expressed in terms of molarity refer to the concentration of the 23 833- M_r polypeptide unless otherwise indicated.

Stopped assay of enzyme activity based on the u.v. absorbance of the enol of IAP

Assays at 30 °C were in 0.1 M Hepes buffer, pH 7.0, containing 0.2 mM of freshly added $MnCl_2$ (unless otherwise stated), were started with either enzyme or IGP and were stopped with alkali (to 1.2 M NaOH) to obtain time points between 0 and 2 min. After 90 min the absorbance was read at 290 nm. Starting concentrations of IGP were sufficiently in excess of K_m for there to be no significant curvature in the progress curve (Figure 5 below), and initial rates were estimated by linear regression through six or more time points. The ϵ_{290} of the enol of IAP was estimated as 6600 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$. One unit of activity was defined as 1 μ mol of IAP formed/min $^{-1}$.

Continuous assay based on coupling to IAPT and glutamate dehydrogenase (GDH)

For the accurate determination of K_m and for other kinetic studies (Hawkes et al., 1993) a sensitive continuous assay method was needed. Potassium Hepes buffer (0.1 M; pH 7.0) contained 0.1 mM pyridoxal phosphate, 0.1 M L-glutamate, 0.1 M NH_4Cl , 0.1 mM $MnCl_2$, 0.20–0.25 mM NADH and a large excess of pure IAPT and GDH (Sigma type III from bovine liver). Assays, at 30 °C in a final volume of 1 ml typically contained 0.1–0.5 μ g of pure Mn-IGPD, 1–5 units (see below) of IAPT and 8–30 units (Sigma) of GDH and were monitored by the decrease in absorbance at 340 nm versus 400 nm in a dual-beam spectrophotometer (cf. Figure 8b below). Dehydration of IGP resulted in quantitative oxidation of NADH up to a ΔD_{340} of 1.0. This was as expected on the basis of the combined equilibrium constants for (i) near-irreversible dehydration of IGP, (ii) a K_{eq} for the formation of histidinol phosphate and 2-oxoglutarate from IAP and L-glutamate of 0.04 (Ames and Horecker, 1956) and (iii) a K_{eq} for the formation of L-glutamate and NAD(P) from NAD(P)H, NH_3 , 2-oxoglutarate and H^+ of 2.22×10^{13} M $^{-1}$ (Frieden, 1963). Using the conventional assay, it was established that the components required for the coupled assay were not, in themselves, significantly inhibitory (the mixture caused < 10% inhibition). At the higher concentrations of the two coupling enzymes, initial lags were negligible, and near-linear rates were maintained for 1 h or more (or until near exhaustion of NADH). The assay was suitable for measuring initial rates in the pH range 6–8 provided that the coupling enzymes were added fresh and kept in sufficient excess for rates to remain independent of their concentration (e.g. higher [IAPT] was necessary at low pH). GDH was in sufficient excess that substrate inhibition by NADH was not a problem, although using NADPH would have permitted the use of less. Assays were started with IGP, since this eliminated an early burst associated with the dilution of enzyme (Figure 6b below).

IAPT was partly purified (essentially as described by Martin and Goldberger, 1967) from *E. coli* DH5 α (pIAPT $\times$ 99). Cells were broken in a French pressure cell in an equal volume of 0.1 M triethanolamine buffer (pH 7.5)/0.1 M NaCl/1 mM EDTA/1 mM dithiothreitol. The extract was centrifuged and treated with protamine sulphate (exactly as described for the

Table 1 Purification of apo-IGPD

Experimental details were as described in the Materials and methods section. Note that the enzyme was not inactivated as a result of precipitation with $(\text{NH}_4)_2\text{SO}_4$. The fractionation was not optimized, and the missing 50% of the activity was almost fully accounted for in the discarded 0–24%-satd.- and > 35%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fractions.

Step	Protein	Protein		Specific activity (units/mg)	Total activity (units)	Yield (%)
		Concn. (mg/ml)	Total (mg)			
1	Crude extract	16.3	17 278	4.8	82 781	100
2	24–35%-(NH_4) ₂ SO ₄ pptn.	13.7	1370	27.3	37 434	45
3	Sephadex G-50 gel filtration of fraction no. 2	8.6	1720	22.6	38 828	47
4	Q-Sepharose gradient					
5	Front IGPD band	14	560	42.4	23 737	29
6	Rear IGPD band	10.8	216	45.0	9716	12
7	Superdex 200 gel filtration of fractions 4 and 5 combined					
8	Fraction I	1.8	92	55.1	5069	6.1
9	Fraction II	1.2	57	47.5	2707	3.4
10	Fraction III	5.2	435	61.2	26 622	32

purification of apo-IGPD), and the fraction precipitating between 50 and 60%-satd. $(\text{NH}_4)_2\text{SO}_4$ was resuspended and further purified by gel filtration through a column of Superdex 200 equilibrated with 50 mM triethanolamine buffer (pH 7.5)/1 mM dithiothreitol/0.1 M NaCl. The active peak (at M_r 74 000; Henderson and Snell, 1973) was collected and frozen as beads into liquid nitrogen. Typically the protein concentration was 4 mg/ml and its activity about 12 μmol of histidinol phosphate formed/min (units) per mg under the conditions of the coupled assay.

Analysis of proteins for metals and phosphorus

Enzyme fractions for metal analysis were purified by gel filtration through a HiLoad 16/60 column of Superdex 200 and, at the same time, exchanged into a defined buffer [usually 50 mM triethanolamine buffer (pH 7.5)/0.2 M KCl] which had been extracted with Chelex 100. Blanks were samples of buffer collected from the column before the protein. Enzyme samples were completely digested in acid-washed boiling tubes by boiling at 120 °C with 2 ml of 20% H_2O_2 and 6 ml of conc. HNO_3 until only the concentrated acid remained. Great care was taken to ensure that no sample was lost by sputtering of the solution. Samples were then diluted in ultrapure water, and metal analysis was carried out as a commercial service by Southern Science Ltd. (Falmer, Sussex, U.K.) using a Thermo Jarrell Ash ATOMSCAN 25 sequential plasma emission spectrometer equipped with a galvo-driven monochromator operating in a vacuum and thermostatically controlled at 35 °C. The system was calibrated at points between 0 and 4 p.p.m. of analyte (diluted stock solutions from BDH). Phosphate was analysed by the method of Lanzetta et al. (1979), except that Triton N101 was used in place of Sterox detergent.

Purification of apo-IGPD from *E. coli* MM294 (pIGPDX34)

IGPD was purified as the apoenzyme in the presence of EDTA. All buffers and protein purification steps were carried out in the cold at temperatures of 6 °C or less.

Table 1 refers to a typical purification starting from 500 g of frozen cell paste thawed with 500–600 ml of 0.2 M Bistris propane buffer, pH 9.0, containing 0.1 M KCl, 1 mM benzamidine, 25 mM β -mercaptoethanol and 5 mM EDTA. The smooth cell

suspension was disrupted in a French pressure cell and then centrifuged at 30 000 g for 3 h. The supernatant was treated with 0.2% salmine sulphate for 20 min and again clarified by low-speed centrifugation. Protein precipitating between 24 and 35% satn. of $(\text{NH}_4)_2\text{SO}_4$ was collected as a pellet by low-speed centrifugation and resuspended in 50 mM Bistris propane buffer, pH 9.0, containing 5 mM EDTA, 25 mM mercaptoethanol and 0.1 M KCl. The redissolved protein was centrifuged and then filtered through a 1.2 μm -pore-size filter to clarify before exchange through a large (500 ml) column of Sephadex G-50 equilibrated with the same buffer. The protein was then loaded on to a 5 cm-internal-diameter column containing 160 ml of Q-Sepharose, which was developed using a 2 litre gradient between 0 and 1.0 M KCl in the same buffer. Active fractions (obvious as a large double peak on the u.v. trace at 280 nm) eluted between 0.25 and 0.35 M KCl and were normally combined and concentrated by precipitation with 50% $(\text{NH}_4)_2\text{SO}_4$, low-speed centrifugation and resuspension of the pellet in 50 ml of the same buffer containing 0.1 M KCl. The protein solution was then further purified by gel filtration down a column of Superdex 200 equilibrated with 50 mM Bistris propane buffer, pH 9.0, containing 20 mM mercaptoethanol, 2.5 mM EDTA and 0.1 M KCl. The major fractions of protein obvious by u.v. absorbance (cf. Figure 3a) were precipitated by addition of 50% $(\text{NH}_4)_2\text{SO}_4$, collected by low-speed centrifugation and redissolved in 20–30 ml of 50 mM triethanolamine buffer, pH 7.5, containing 0.1 M KCl and 20 mM mercaptoethanol. Protein samples were rapidly frozen as droplets and stored as beads over liquid nitrogen. For 100–150 g of cells, the above procedure was conveniently scaled down to using a 26/10 HiLoad (Pharmacia) column prepacked with 50 ml of high-performance Q-Sepharose and a 16/60 HiLoad (Pharmacia) column of 120 ml of Superdex 200. Other minor variations in procedure included taking a 17–35%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction to improve the yield and, as illustrated in Figure 3(b), sometimes carrying out gel filtration at pH 7.5.

Purification of Mn-IGPD from cells induced in the presence of a high concentration of Mn^{2+}

IGPD was also purified as a metalloenzyme (Mn-IGPD) from cells grown supplemented with additional Mn^{2+} (see above). About 150 g of cells were broken in 0.1 M Bistris propane buffer,

Figure 3 Gel filtration of IGPD through Superdex 200

Chromatograms represent 1–2 ml of protein solution (10–30 mg) loaded on to a 16/60 column (~120 ml volume) of Superdex 200 with the u.v. absorbance at 280 nm (ordinate) recorded versus elution volume (abscissa). The flow rate was 0.5 ml/min. All buffers were extracted with Chelex 100. Chromatogram a represents gel filtration as in the normal course of purification (but on a smaller scale) in Bistris propane buffer, pH 9.0, containing 0.1 M KCl and 2.5 mM EDTA. Three u.v. peaks corresponding to fractions I, II and III (cf. Table 1) of apo-IGPD activity were resolved. Chromatogram b was the same material but chromatographed in 50 mM triethanolamine buffer, pH 7.5. Chromatogram c was an experiment in which the major peak of apo-IGPD, III, was collected, concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$, redissolved in 50 mM triethanolamine buffer, pH 7.5, containing 0.2 M KCl, and then re-run in the same buffer. Trace d represents an experiment identical with that producing trace c, except that the concentrated apo-IGPD was first pre-exchanged down Sephadex G-25 into 25 mM triethanolamine buffer, pH 7.5, containing no KCl. Chromatogram e was, again, from an experiment identical with that producing trace c, except that, in this case, 2 mM MnCl_2 (about 5-fold molar excess over protomer) was incubated with the concentrated enzyme for 5 min prior to chromatography. There was no measurable loss of enzyme activity in any of these experiments. M_r calibration was on the basis of BSA (66 000), yeast alcohol dehydrogenase (150 000), sweet-potato β -amylase (200 000), horse spleen apoferritin (430 000) and bovine thyroglobulin (669 000) supplied as a standard kit from Sigma.

pH 9.0, containing 10 mM 1,2,4-triazole, 10 mM K_2HPO_4 , 0.1 M KCl and 40 mM β -mercaptoethanol. After removal of the precipitate formed with salmine sulphate, the fraction precipitating between 0 and 11% of poly(ethylene glycol) 3350 was collected by low-speed centrifugation, redissolved in the same buffer and then diluted 2-fold with water and filtered before loading on to a 26/10 HiLoad column of high-performance Q-Sepharose equilibrated with 50 mM Bistris propane buffer, pH 9.0, containing 10 mM 1,2,4-triazole, 10 mM K_2HPO_4 and 40 mM β -mercaptoethanol. A linear 800 ml gradient was developed between 0 and 1.0 M KCl, and the active fractions were combined and concentrated by centrifugal ultrafiltration through a Centriprep 30 (Amicon) membrane before gel filtration down a 16/60 column of Superdex 200 equilibrated with the same buffer containing 0.1 M KCl. The peak of assembled Mn-IGPD was obvious by u.v. monitoring and was eluted in the expected position (cf. Figure 3e). Again the protein solution was beaded and stored as frozen droplets over liquid nitrogen.

RESULTS AND DISCUSSION

Corrections to the *HIS3* gene sequence of *S. cerevisiae*

Prior to gene-expression studies, the IGPD-coding region in pUC8Sc2676 was re-sequenced. The resolution of one particularly bad compression using dITP revealed six changes to the sequence published by Struhl (1985). These changes resulted in the leucine residue at position 109 becoming glycine and the introduction of an additional valine at position 111 (numbered according to the original published sequence). The electrospray mass spectrum of the polypeptide confirmed that the altered sequence was correct (Figure 4).

Purification of IGPD

The methods described above were guided by pilot experiments that indicated that IGPD activity was stable for weeks in the

Figure 4 Mass spectra of Mn-IGPD

(a) Depicts the laser-desorption mass spectrum of Mn-IGPD. % on the ordinate scale refers to the sum total of the peaks. The mass ion (MH^+) peak determined at 23834 is reasonably close to the calibration standard (carbonic anhydrase at 29022.7); the lesser peaks are less accurately determined and represent the 0.5-, 2- and 3-fold multiples of the mass ion. (b) Is the electrospray mass spectrum of Mn-IGPD transformed to a mass scale. In this case, % is referenced to the highest peak.

cold-room at pH 9.0 in the presence of EDTA or, at pH values near 7, in the presence of Mn^{2+} and a combination of 10 mM phosphate and 10 mM 1,2,4-triazole, the latter being competitive inhibitors (Wiater et al., 1971). It was noted that precipitation with $(NH_4)_2SO_4$ resulted in a large loss of activity unless EDTA was also present (indicating that the enzyme was probably inactivated by trace-metal contaminants). Because of the tendency of $Mn(OH)_2$ to precipitate and then further oxidize, Mn^{2+} was not added to solutions at high pH. Accordingly, alternative strategies were adopted for purifying the enzyme as either (1) an apo form in the presence of EDTA or (2) as a stable metalloenzyme, Mn-IGPD.

Apo-IGPD

Purification of apo-IGPD

The purification described in the Materials and methods section is summarized in Table 1. While not optimized for yield [half the activity was discarded at the $(NH_4)_2SO_4$ step], there was no significant loss of activity at any step, and the activity of the pure enzyme was therefore unlikely to have been significantly reduced by inactivation during the course of purification.

Three different forms of apo-IGPD

Gel filtration down Superdex 200 yielded three fractions of apo-IGPD which, by SDS/PAGE (Figure 2) were all substantially pure. The major of these, fraction III, was eluted in a volume corresponding to an M_r of about 70000. This estimate of M_r was also consistent with the particle radius (3.7 nm) measured by laser light scattering using a model 801 dynamic-light-scattering instrument from Protein Solutions, High Wycombe, Bucks., U.K. Thus the bulk of the apo-IGPD appeared to be present as a trimer of the 23833- M_r polypeptide.

The minor fractions I and II appeared in variable amounts (note that Table 1 and Figure 3a refer to different preparations which yielded different relative amounts of fractions I and II). They appeared to interconvert and shift the M_r according to pH. Reducing the pH from 9.0 to 7.5 caused a decrease in the amount of fraction II and a corresponding increase in fraction I. Fraction I was also eluted earlier from the column than it was at pH 9.0 (Figures 3a and 3b). On the basis of gel filtration at pH 7.5, the M_r values of fractions I and II were estimated to be 500000 and 220000 respectively.

Apo-IGPD was eluted as a double peak from Q-Sepharose, which, in the normal course of purification, was recombined before further purification by gel filtration (Table 1). Experiments in which the front and back peaks from Q-Sepharose were treated separately revealed that fractions I and II were associated with the trailing peak. Thus the higher- M_r forms of apo-IGPD were eluted from Q-Sepharose later than the major form, fraction III.

Properties of apo-IGPD

As normally assayed (with an excess of Mn^{2+} included in the assay) all three M_r fractions of apo-IGPD were similarly active (Table 1).

IGPD from *S. cerevisiae* includes eight phenylalanine residues, only a single tyrosine residue (position 75) and a single tryptophan (position 139) residue in a polypeptide chain of 220 amino acids (Struhl, 1985; the present study). The u.v.-visible spectrum of apo-IGPD provided no evidence for additional chromophores. The absorbance of a 1 mg/ml solution at 280 nm was about 0.4 in a 1-cm-pathlength cell. The maximum for fluorescence ex-

citation was at 282 nm and the emission maximum at 330 nm. Thus the single tryptophan residue is probably embedded within the protein core. Careful analysis of apo-IGPD indicated no significant level of phosphorus (< 0.07 atom per 23833- M_r protomer) or any of a range of metals (< 0.02 Mn, < 0.04 Fe, < 0.04 Zn, < 0.02 Cd, < 0.07 Cu, < 0.07 Ni and < 0.04 Co atom per 23833- M_r protomer) in either the low- (fraction III) or high- (fraction I) M_r forms of apo-IGPD.

Salt-dependent assembly of apo-IGPD (fraction III) to the high- M_r form (fraction I)

As isolated in buffer containing EDTA and KCl, apo-IGPD was stable as the low- M_r form, fraction III. There was no indication of conversion into any other form when it was concentrated and rechromatographed under the same conditions (Figure 3c) or in response to changing the pH between 7.5 and 9.0. However, following exchange into buffer lacking KCl it slowly shifted to a high- M_r form (500000 at pH 7.5). This change was reported by a correspondingly gradual quenching in the fluorescence emission of the single tryptophan residue at 330 nm. Dilute solutions (0.1 mg/ml) of apo-IGPD in 50 mM triethanolamine buffer/0.2 M KCl (extracted with Chelex 100) fluoresced steadily. Following exchange into buffer lacking KCl there was a small and gradual decline in fluorescence (5–10% over the course of 1 h or more). The higher oligomer did not revert to the 70000- M_r form following exchange back into KCl-containing buffer (Figure 3d). The catalytic activity of the enzyme (assayed in the presence of excess Mn^{2+}) did not change as a result of the increase in M_r . Thus transfer to a low concentration of salt resulted in the slow and irreversible conversion of the low- M_r form, fraction III, of apo-IGPD to a form which was indistinguishable from fraction I. This process of assembly was clearly distinct from that (see below) which occurred in specific response to the addition of Mn^{2+} , since (1) fraction I contained no significant amount of metal (see above) and (2) assembly was not prevented by the addition of EDTA and therefore was not due to the scavenging of bivalent cations.

Dependence of apo-IGPD on metal ions for catalytic activity

It was observed early on (Ames, 1957) that IGPD was most active in the presence of Mn^{2+} ions, and this has since been a routine addition to assays. The optimal requirement for concentrations in excess of 0.1 mM would seem to imply a relatively labile and weak association between the protein and metal ion. However, since IGPD has not previously been isolated in a defined form with a defined metal content, the interaction of the protein with Mn^{2+} has not previously been studied.

The apo form of IGPD described here contained no significant amount of metal and was normally assayed in the presence of 0.2 mM Mn^{2+} . However, as much as a half of the maximum activity was sometimes observed when no metal ion was added to assays. Such metal-independent activity would obviously be very significant if it could eliminate a direct role for the metal in catalysis and was therefore investigated further. As a percentage of the activity obtained with Mn^{2+} , 'metal-independent' activity was noted to decrease with increasing concentrations of enzyme (it was less than 5% at concentrations of enzyme protomer above $2 \mu M$). Furthermore, there was always a significant lag before it reached a maximum rate. The lag became much longer, and metal-independent activity was minimized when the enzyme and assay solutions were pre-extracted with Chelex 100. Thus, for example, a $6 \mu M$ solution of (fraction III) apo-IGPD (pre-exchanged into EDTA-free buffer and extracted with Chelex

100) appeared inactive during the first minute or so after it was added to an assay solution of Hepes buffer, pH 7.0, containing IGP (also pre-extracted with Chelex 100). Thereafter, activity slowly increased until, still increasing after 30 min, it was about 1.0 unit/mg (corresponding to 2% of the activity with Mn^{2+} added). All activity was abolished with the addition of 5 mM EDTA.

From these observations it was concluded that a bivalent-metal ion was indeed essential for catalytic activity and that the apparent 'metal-independent' activity must have been due to the ability of the apoenzyme to scavenge even traces of those bivalent-metal cations which support catalytic activity. Apo-IGPD was activated by a range of metals other than Mn^{2+} , including Co^{2+} , Fe^{2+} , Ni^{2+} , Zn^{2+} and Cd^{2+} (T. R. Hawkes and L. S. Edwards, unpublished work). Since all of these were at or below their detection limits in the Chelex-extracted assay solution their (presumed) scavenging by apo-IGPD would require dissociation constants for the corresponding complexes between protein and metal ion in the range less than 1.0 μM .

Mn^{2+} -induced assembly of apo-IGPD to a high- M_r metalloenzyme (Mn-IGPD)

A 4–5-fold molar excess of MnCl_2 (per 23833- M_r polypeptide) was added to the 70000- M_r form of apo-IGPD (fraction III) maintained, as described above, in buffer containing KCl. Gel filtration through Superdex 200 in the same buffer indicated that this treatment had caused the bulk of the protein to shift to a form with an M_r near 500000 (Figure 3e). Unlike fractions I and II of apo-IGPD, the M_r of the enzyme assembled with Mn^{2+} did not shift with pH. Similarly to the oligomerization of apo-IGPD at low concentrations of salt, Mn^{2+} -induced assembly was indicated by a slight, but definite, quenching (about 5%) of fluorescence emission at 330 nm (but, again, with no shift in the wavelength of the maximum). In this case, quenching occurred rapidly, indicating that Mn^{2+} -induced assembly was complete within seconds at micromolar concentrations. Even with a large excess of Mn^{2+} , some (10–20%) of the apo-IGPD appeared to remain unchanged. The material which remained in the trailing fraction was assayed and found to be inactive (but was still mainly IGPD on the basis of SDS/PAGE). The Mn^{2+} -induced shift in M_r was therefore a specific property of active apo-IGPD and, potentially therefore, an ideal way of obtaining highly pure IGPD free of inactive polypeptides. The enzyme assembled from apo-IGPD with Mn^{2+} was a catalytically active metalloenzyme (Mn-IGPD) within which the metal remained tightly bound. Its characterization is further described below.

Mn^{2+} induced apo-IGPD to assemble to a stable high- M_r metalloenzyme, whereas Mg^{2+} did not. This specific ability of Mn^{2+} ions strongly suggests that the metal has a structural role in the assembly of the active oligomer. However, it would also seem likely that the metal has some role in catalysis since, of itself, assembly was not sufficient for enzyme activity. In metal-free assay solutions or in assay solutions containing EDTA, the high- M_r form of apo-IGPD, fraction I, was no more active than the low- M_r form, fraction III. The isolation of apo-IGPD described here promises to facilitate further exploration of the role of the metal through the assembly and characterization of IGPD containing different metal ions.

Mn-IGPD

Purification

The catalytically active metalloenzyme Mn-IGPD was obtained in two different ways. Either, as described above, it was assembled

in vitro by addition of Mn^{2+} ions to apo-IGPD and then isolated as the leading peak from gel filtration through Superdex 200 (cf. Figure 3e) or it was purified through as Mn-IGPD from cells grown in a medium supplemented with MnCl_2 . For the latter, cells were grown on a medium which was low in any of the metal ions which might compete with Mn^{2+} and, just prior to induction, was supplemented with a high concentration of Mn^{2+} . The cells were then broken and Mn-IGPD purified to homogeneity in buffer solutions which contained neither Mn^{2+} nor EDTA and with poly(ethylene glycol) used as precipitant in place of $(\text{NH}_4)_2\text{SO}_4$. Again, there was no significant loss of activity at any step, and a similar level of induction and final yield of IGPD was achieved as in the purification of apo-IGPD. Mn-IGPD behaved differently from apo-IGPD in precipitating at lower concentrations of poly(ethylene glycol) and, as expected, was eluted earlier from gel filtration through Superdex 200. It was impossible to prove that assembly and incorporation of Mn^{2+} had really taken place *in vivo* and not *in vitro* through scavenging of Mn^{2+} immediately following cell breakage. However, strong evidence was provided by the growth characteristics of the cells following induction. In the absence of Mn^{2+} , growth virtually stopped (presumably largely because the accumulation of apo-IGPD withdrew essential bivalent-metal cations from inside of the cell), whereas, in its presence, growth continued almost without pause.

Properties of Mn-IGPD

Wherever they were examined, the properties of Mn-IGPD assembled *in vitro* or *in vivo* were identical. Unless otherwise indicated, the results below were obtained from the enzyme assembled *in vitro*.

The u.v.-visible and fluorescence spectra of Mn-IGPD were not significantly different from those of apo-IGPD. Mn-IGPD assembled *in vitro* contained $1.35 \pm 0.15 \text{ Mn}/23833\text{-}M_r$ protomer. Similarly, Mn-IGPD assembled *in vivo* contained $1.41 \pm 0.1 \text{ Mn}$ and no other detectable metal (less than 0.02 atom of Co, Ni or Cd). The non-stoichiometric content of Mn^{2+} was peculiar, but was confirmed in several repeat analyses. This metal was clearly very tightly bound to the protein, since the analysis remained consistent between preparations of Mn-IGPD, assembled and purified in quite different ways and following successive steps of exchange into metal-free buffer via gel filtration.

On the basis of gel filtration, the M_r of Mn-IGPD was about 500000. This estimate of M_r was also consistent with the particle radius (7.2 nm) measured in solution by laser light scattering using the model 801 dynamic-light-scattering instrument. Low-angle X-ray precession photographs of a number of different crystal forms were all consistent with 432 symmetry (K. W. Wilkinson and D. W. Rice, unpublished work). Mn-IGPD was therefore an oligomer of 24 subunits with a total M_r , calculated on this basis, of 573000. Mano et al. (1993) suggested that the wheat enzyme also consists of 24 subunits, although in this case the evidence was based only on gel filtration.

Given the large shift in M_r relative to fraction III of apo-IGPD, Mn-IGPD assembled *in vitro* and further purified by gel filtration might have been expected to be fully pure. Indeed, as normally assayed in the presence of additional Mn^{2+} , the activity of Mn-IGPD was consistently about 20%, higher than that of apo-IGPD, presumably because of the efficient removal of inactive polypeptides at the gel-filtration step. However, SDS/PAGE indicated that low- M_r contaminants remained (Figure 2). Further investigation by N-terminal sequencing of blots from SDS-containing gels indicated that at least some of

Figure 5 Conversion of IGP into IAP: time course with and without β -mercaptoethanol

Assay mixtures in 0.1 M HEPES, pH 7.0, contained 0.5 mM MnCl_2 , 3.5 mM IGP (nominal concentration) and either 0.1 M β -mercaptoethanol (□) or no mercaptoethanol (▽). Assays were started with the addition of about 12 μg of Mn-IGPD to a final volume of 80 μl . The broken line is a theoretical progress curve based on the measured K_m of 0.1 mM. The continuous line is an example of a fit to a K_i value of 0.2 mM for competitive inhibition by the product, IAP.

these derived from proteolysis. Thus a faint higher- M_r band (probably a dimer) and a minor band of M_r close to 22000 detected in SDS-containing gels of the protein from redissolved crystals of Mn-IGPD were found to share the same N-terminal sequence (Met-Thr-Glu-Gln-Lys-Ala-Leu) as IGP (Struhl, 1985). The N-terminus of a band of M_r close to 15000 was Ala-Glu-Gln-Ala-Thr-Gln-Ser (the sequence of IGP starting from amino acid 44). Thus this too was derived from IGP, with, in this case, cleavage having occurred after the valine residue at position 43. It seemed likely, therefore, that most of the faint low- M_r bands derived from proteolysis. This was a particular concern, since even small amounts of such peptide fragments could, in principle, create sufficient disorder to limit the X-ray diffraction of protein crystals. A number of combinations of different ways of growing the cells and of purifying IGP were investigated to see if proteolysis could be minimized. IGP was re-cloned for overexpression at 37 °C using an IPTG-inducible promoter. Apo-IGPD was purified through in the presence of a cocktail of proteinase inhibitors including EDTA, EGTA, leupeptin, pepstatin and 4-(2-aminoethyl)benzenesulphonyl fluoride (a serine-proteinase inhibitor from Pentapharm, Basel, Switzerland). These experiments are not reported in detail because, in all cases, Mn-IGPD was essentially the same and was always proteolysed in the same way and to about the same extent. Given the precautions taken during purification it seems probable that proteolysis took place during the course of expression in *E. coli*.

The molecular ion (MH^+) detected by 'time-of-flight' laser-desorption m.s. was at 23834 and in good agreement with the major peaks at 23831.2 ± 1.1 and 23703.9 ± 0.8 detected by electrospray m.s. (Figure 4). These species could be readily accounted for as the mass ion based on the complete sequence (23833.3 after accounting for natural isotope abundances) with about 50% loss of a single methionine residue. Since there is a methionine residue at either end, this could, in principle, have been lost from either the C- or the N-terminus. The detection of a near-even mix of peptides with N-termini sequencing as either Met-Thr-Glu-Gln-Lys-Ala-Leu etc. or Thr-Glu-Gln-Lys-Ala-Leu etc. confirmed that methionine was lost from the N-terminus. A series of minor peaks with M_r increments of 60 above the two

major peaks were also evident. These may have arisen as non-covalent adducts with a trace of acetic acid. The 2-unit shortfall in the value for the mass ion peak may be significant or could have arisen from a slight calibration error or a distortion due to the proximity of one of the presumed adduct peaks.

Thus, consistent with the featureless u.v.-visible spectrum and lack of phosphorus, electrospray m.s. indicated that Mn-IGPD contained an unmodified polypeptide chain and no covalently attached cofactor.

Progress curve for the conversion of IGP into IAP

Figure 5 describes a complete progress curve for the conversion of IGP into IAP. This provided a useful indication of the appropriate region for initial-rate measurements and, via the computer fit based on the known K_m (below), the first indication of the extent to which this enzyme is inhibited by the product, IAP. The enzyme was stable under assay conditions, and lability would not have contributed to curvature over the time course indicated. The dotted line describes the shape of the curve expected purely on the basis of the measured K_m (see below) of 0.1 mM for IGP. Clearly this was a poor fit to the data. The continuous line is an example of a computer fit based on the assumption of competitive inhibition by the product, IAP. Almost as good a description could be obtained taking into account only the possibility of competitive inhibition by the (non-substrate) *threo* diastereomer present in the preparation of IGP. However, at only 15% of the total IGP, it was necessary to assume that this was a very potent inhibitor indeed to affect the curvature as much as observed. It seems probable that most of the inhibition originates from IAP ($K_i \geq 0.2$ mM) with some contribution from *threo*-IGP.

The K_m for IGP and the K_i for inhibition by 1,2,4-triazole

Using assays based on the u.v. absorbance of the enol of IAP, the K_m for IGP of IGP from *S. cerevisiae* has previously been estimated as between 0.30 and 0.54 mM (Wiater et al., 1971; Glaser and Houston, 1974). However, it is notoriously difficult to measure initial rates using stopped assays, and K_m values tend to be overestimated wherever limitations in assay sensitivity dictate that detectable changes in substrate concentration need correspond to a significant fraction of the K_m value itself. Therefore the continuous assay, described here, was used to obtain more accurate estimates of the K_m value for D-erythro-IGP and of the K_i value for competitive inhibition by 1,2,4-triazole of 0.10 ± 0.02 mM and 0.12 ± 0.02 mM respectively at pH 7.0. The K_m value takes into account the estimated 85% purity of the substrate enantiomer. Depending on its potency as a competitive inhibitor, the expected effect of the 15% of non-substrate, *threo*, diastereomer would be to reduce the observed value of the K_m relative to that for the pure substrate enantiomer (Segal, 1975). Data were computer-fitted directly to the Michaelis-Menten equation by non-linear regression and least-squares fitting as described by Duggleby (1981).

Given the relative simplicity of its structure, 1,2,4-triazole competes remarkably effectively with IGP. The synergism of inhibition observed in mixture with phosphate supports the view that it only makes binding interactions with the 'heterocyclic' end of the substrate-binding site in the enzyme (Wiater et al., 1971). Its potency most probably derives from its resemblance to a diazafulvene intermediate in the catalytic reaction (Hawkes et al., 1993).

Figure 6 Effects of enzyme dilution and of the order of addition on activity

(a) Collates specific activities determined over a wide range of enzyme concentrations in continuous (+) and stopped assays (□), which all contained more than 2 mM IGP. (b) Open squares (□) show the time-dependence of IAP formation in assays in 50 mM Hepes, pH 7.0, containing 10 mM IGP, 0.1 M β-mercaptoethanol and 0.5 mM MnCl₂, which were started by addition of 0.14 μg of Mn-IGPD to a final volume of 80 μl (70 nM protomer). The initial stock solution of Mn-IGPD was about 1.2 μM. Closed circles (●) show a similar dependence, except that assays contained only 0.1 μg of Mn-IGPD (50 nM protomer) and were started by the addition of a small volume of concentrated IGP.

Dependence of activity on enzyme concentration

Figure 6(a) summarizes the results of many experiments in which the activity of Mn-IGPD was measured over a wide range of enzyme concentrations. While the scatter was quite wide (most probably largely reflecting poor accuracy in some protein determinations), the trend was obvious. Activity was highest at enzyme concentrations in excess of 1.0 μM. At very low concentrations, activity did not tend to zero but to a limit at just below a half of that observed at high enzyme concentrations. When very low concentrations of Mn-IGPD were used (as in the coupled assay), activity remained near linear for at least 1 h. At very high concentrations it was only possible to judge linearity over the first few minutes (the substrate being too quickly consumed). When assays were started by the addition of enzyme and, in the process, the enzyme was diluted from a relatively high concentration to less than 0.1 μM, there was always an apparent burst of activity over the first few minutes. This did not occur

Figure 7 Titration of Mn-IGPD with 2-hydroxy-3-(1,2,4-triazol-1-yl)propylphosphonate in the presence and absence of additional Mn²⁺ ions

The Figure summarizes the specific activities measured in a series of assays in 50 mM Hepes buffer, pH 7.0, which contained 70 μg of Mn-IGPD/ml (i.e. ~2.9 μM Mn-IGPD protomer as indicated with an arrow) and either 0.5 mM MnCl₂ (□) or none (●). Concentrations of inhibitor were as indicated. After 5–10 min pre-incubation of the diluted enzyme with the other assay components, assays were started with 7.5 mM IGP.

when the enzyme was first diluted into the assay solution, left for 5 or more min and the assay started with addition of IGP (Figure 6b). It was therefore better to start assays with IGP to obtain initial rates for the evaluation of kinetic constants. The above data are most easily understood in terms of a model in which Mn-IGPD slowly ($t_{1/2}$ 2–3 min) dissociates to a stable, but less active, form on dilution. Gel-filtration studies of dilute solutions of Mn-IGPD monitored by u.v. indicated that the high- M_r 24-subunit form was retained down to concentrations at least as low as 200 nM of the 24-mer (i.e. 5 μM protomer). The observed dependence of activity on enzyme concentration suggested a K_d value of around 10 nM for the dissociation of the 24-mer to a less active lower oligomer. It is not clear that this should have any functional or regulatory significance. Single polypeptide enzymes seem, most often, to function as oligomers (Goodsell and Olsen, 1993) and there are other examples of enzymes (cf. chorismate synthase from *E. coli*; Ramjee et al., 1994) also not at obvious regulatory points in metabolism and where dissociation of the active oligomer appears to regulate activity over a quite similar range of protein concentrations.

Titre of active sites and catalytic turnover rate of Mn-IGPD

Figure 7 shows the effect on activity of titrating Mn-IGPD with increasing concentrations of 2-hydroxy-3-(1,2,4-triazolyl-1-yl)propylphosphonate, a very potent (K_i 0.6 nM at pH 7.0) and slow dissociating (k_{off} 0.04 min⁻¹) inhibitor of IGP (Hawkes et al., 1993). Although the inhibitor was racemic, experiments using ¹⁴C-labelled material indicated that both enantiomers bound very tightly ($K_d < 50$ nM) to Mn-IGPD. Thus, at the concentration of enzyme (3.0 μM) used in the experiment described, all of the inhibitor would have been expected to bind stoichiometrically. From a number of experiments of similar design, it was estimated that there were 0.9 ± 0.1 inhibitor binding sites per single molecule (23833- M_r protomer) of enzyme. The binding of the inhibitor to Mn-IGPD was also reported by a slight (5%), but definite, quenching in the fluorescence emission at 330 nm. In 50 mM triethanolamine buffer, pH 7.5, containing 0.2 M KCl, maximal quenching of the fluorescence of a 4 μM solution of Mn-IGPD required the addition of between 3 and 4 μM inhibitor. In control experiments the inhibitor had no effect on the fluorescence of either apo-IGPD or of Mn-IGPD after it was first inactivated by addition of EDTA. These data were consistent with a stoichiometry of between 0.75 and 1.0 inhibitor binding sites per protomer of active enzyme.

The maximum activity of about 80 μmol of IAP formed/min per mg at 30 °C (Figure 6a) was much greater than has previously been reported for an IGP from any source. The fact that purification was achieved with little loss of activity and that similar material was obtained via a number of different routes suggested that this activity was close to its limiting value. This was confirmed by the near-unity titre of binding sites for 2-hydroxy-3-(1,2,4-triazol-1-yl)propylphosphonate, an inhibitor which most certainly derives its potency from its close resemblance to a high-energy reaction intermediate in catalysis (Hawkes et al., 1993). Thus, with one 'active' site per protomer, the maximum catalytic turnover rate of the enzyme at 30 °C was calculated as about 32 s⁻¹.

Dependence of activity on additional Mn²⁺ ions

Mn²⁺ has long been recognized as a requirement for IGP and is routinely included in the solution used to assay the enzyme. The results above were, in general, obtained with 0.2 mM MnCl₂ included in the assay buffer. However, having obtained IGP in a form, Mn-IGPD, in which this metal appeared to be stably

Figure 8 Time course of Mn-IGPD activity: effect of Mn^{2+} and of Mg^{2+}

In (a), assay mixtures contained $\sim 2.6 \mu\text{g}$ of Mn-IGPD in a final volume of $80 \mu\text{l}$ of 50 mM Hepes buffer, pH 7.0 (▽) which also contained 0.5 mM MgCl_2 (+) or 0.5 mM MnCl_2 (□) and were started with the addition of 8 mM IGP and stopped with 1.25 ml of NaOH. The activity was ~ 66 units/mg with Mn^{2+} and ~ 32 units/mg without. (b) represents coupled assays which did (lower trace) or did not (upper trace) contain 0.2 mM MnCl_2 . Reactions were started by adding $\sim 0.7 \mu\text{g}$ of Mn-IGPD to a final volume of 1.05 ml, followed, after 1 min, by the addition of 1 mM IGP. Initial rates with and without the addition of Mn^{2+} were 23 and 15 units/mg respectively.

incorporated into the protein, we expected that Mn^{2+} ions should no longer be a requirement for activity. This was indeed the case, and Mn-IGPD was highly active (up to $40 \mu\text{mol}/\text{min}$ per mg) in solutions containing only buffer and IGP. Activity was completely inhibited by the addition of 5 mM EDTA, showing, as expected, that the intrinsic metal was essential for catalysis.

The strength of the association between the metal and the protein in Mn-IGPD was examined through a study of the effect of time and dilution on activity and its dependence on the addition of Mn^{2+} ions to the assay solution. As shown in Figure 8(a), activity started immediately, without any apparent lag or burst, on the addition of enzyme and continued steadily. The same activity was observed when assays were started with IGP. Thus Mn-IGPD did not appear to partly dissociate into Mn^{2+} and inactive apoenzyme immediately following dilution into the assay (in the experiment described, dilution was from an initial stock solution of about $25 \mu\text{M}$ to a final concentration of $1.3 \mu\text{M}$). When the concentration of enzyme in the final assay was further reduced to 25 nM or below and assays were run for longer, activity was observed (Figure 8b) to decline by about half over the course of 15 min (or slower with some preparations of enzyme). Since this loss of activity was entirely prevented (and activity stimulated) by the specific addition of Mn^{2+} to the assay solution (Figure 8b), it was taken to correspond to the rate of loss of the metal ion from Mn-IGPD. The position of the equilibrium governing this loss would be expected to depend on the concentrations and dissociation constants associated with all the other Mn^{2+} -binding species in the assay solution as well as the concentrations of other competing metal ions. On the basis of e.p.r. linewidth broadening, the dissociation constant of IGP from Mn^{2+} in solution at pH 7.5 was determined to be about 5.5 mM (i.e. typical of phosphate esters; Townsend, 1954). Accordingly, in the absence of Mn^{2+} and at very high concentrations of IGP (50 mM), it was possible to generate assay time courses which were markedly more curved than shown in Figure 8(b) (where [IGP] was 1 mM).

Overall, these data were consistent with the notion that Mn-IGPD is a stable metalloenzyme which only slowly loses its metal at high dilutions. This view also accords with the evidence, given above, that Mn-IGPD retained a consistent metal content through purification and that the apoenzyme was capable of scavenging bivalent-metal ions at submicromolar concentrations. It was therefore surprising to discover that the activity of Mn-

Figure 9 Stimulation of activity by Mn^{2+} : dependence on Mn^{2+} concentration

Assays were similar to those in Figure 8(a), except that the final pH was 6.6 and concentrations of Mn^{2+} were varied as indicated (ordinate = $\log [\text{Mn}^{2+}]$). Percentage of maximum stimulation (abscissa) was calculated as $100 \times (\text{activity observed} - \text{activity}_0) / (\text{activity}_{\text{max}} - \text{activity}_0)$ where activity_0 is the activity observed without added Mn^{2+} and $\text{activity}_{\text{max}}$ that at a saturating concentration (i.e. within a few per cent of the activity observed at 0.5 mM Mn^{2+}). □, Data from reactions started with Mn-IGPD; +, data from reactions started with the addition of IGP.

IGPD nearly doubled when high concentrations of Mn^{2+} were added to the assay solution. As shown in Figures 8 and 9, the effect was immediate, ion-specific (Mg^{2+} had no effect up to 10 mM) and was associated with a relatively high K_s value for half-maximal stimulation of about $20 \mu\text{M}$ Mn^{2+} . Thus, in contrast with the stable initial incorporation of Mn^{2+} into Mn-IGPD, some weaker and more labile association between protein and metal ion was implicated. It therefore seems probable that the stimulatory effect of Mn^{2+} on the activity of Mn-IGPD involves binding at a different site from that involved in assembly. However, the specificity for Mn^{2+} over Mg^{2+} in both cases is noteworthy.

We carried out a number of experiments to try and understand the basis of the 2-fold increase in activity. As shown in Figure 8, the titre of binding sites for 2-hydroxy-3-(1,2,4-triazol-1-yl)propylphosphonate did not increase with the addition of Mn^{2+} ions. Thus no new catalytic sites were created. Aside from a shift in the pH-dependence of k_{cat} (discussed below under that heading), nor were there any significant changes in kinetic parameters which might have reported on the changed function of existing sites. The measurement of initial rates in the absence of Mn^{2+} posed problems because of the confounding effects of the slow loss of Mn^{2+} from Mn-IGPD following dilution (Figure 8b) and the need to avoid the initial burst associated with dilution of the enzyme (Figure 6b). Furthermore, in the absence of Mn^{2+} , there seemed the possibility of substrate inhibition due to the chelation of Mn^{2+} by IGP (associated with a K_d of about 5.5 mM). The latter was shown not to be significant over the range of concentrations (up to 2 mM) used to determine the K_m . Finally, a K_m value for IGP of 0.09 ± 0.02 mM and a K_i value for 1,2,4-triazole of 0.09 ± 0.03 mM were determined. These were not significantly different from the values obtained (above) in the presence of Mn^{2+} .

The degree of stimulation observed with the addition of Mn^{2+} ions did not appear to depend upon the concentration of enzyme in the assay (and, *vice versa*, nor did the dependence of activity on protein concentration vary with $[\text{Mn}^{2+}]$). Thus the effects of Mn^{2+} and of protein concentration on activity were separate and not, for example, different effectors of the same equilibrium

Figure 10 pH profile for the k_{cat} of Mn-IGPD: effect of Mn^{2+}

Assay mixtures contained 10 mM IGP in a mixture of 0.2 M Taps, 0.2 M Bistris propane and 0.4 M acetate buffer (□) and were adjusted with KOH to achieve near constant ionic strength across the range of pH (cf. Ellis and Morrison, 1982). ▽, ▲, Experiments in which 0.18 mM MnCl_2 (freshly added) was included; ▽, buffer one-quarter strength; ▲, buffer strength as described above. Reactions were started by addition of about 0.5 μM Mn-IGPD and were stopped at various times up to 70 s. On this time scale the enzyme was fully stable across the range of pH. Although the K_m for IGP increased at low values of pH (e.g. about 0.4 mM at pH 6.0), 10 mM IGP was enough to obtain a good estimate of k_{cat} across the range (similar values were obtained at the extremes of pH using 20 mM IGP). The lines represent least-squares fits to the equation describing the concentration of the singly protonated species in a doubly ionizing system, i.e.:

$$k_{\text{cat}}(\text{pH})/k_{\text{cat}}(\text{maximum}) = K_2[\text{H}^+]/(1 + K_2[\text{H}^+] + K_1K_2[\text{H}^+]^2)$$

Without Mn^{2+} , the best fit was to bell-shaped curves defined by apparent $\text{p}K_a$ values at 6.45 and 7.95 and, with Mn^{2+} , to values at 5.75 and 7.8 (the $\text{p}K_a$ values being minus the logarithm of the two proton dissociation constants, K_1 and K_2).

governing a transition between a more and a less active form of the enzyme.

Dependence of activity on pH

Figure 10 shows the pH-dependence of k_{cat} , using relatively high concentrations of enzyme (0.5 μM) in the stopped assay. With Mn^{2+} ions included, the data were fitted to a bell-shaped curve with $\text{p}K_a$ values at 5.75 and 7.8. These values appeared relatively insensitive to changes in ionic strength and to the particular mixture of buffers used. They were also in close agreement with $\text{p}K_a$ values of 5.8 and 7.7 fitted to data obtained using much lower concentrations of enzyme in the coupled assay (Hawkes et al., 1993). Thus, whatever mechanism is responsible for the reduction in activity at low concentrations of enzyme (Figure 6a), the lack of a shift in the $\text{p}K_a$ values would suggest that the same events within the catalytic cycle govern k_{cat} . In the absence of Mn^{2+} , the lower $\text{p}K_a$ was markedly shifted and the curve was best fitted to an optimum at 7.2 with $\text{p}K_a$ values at 6.45 and 7.95. The wider separation of $\text{p}K_a$ values in the presence of Mn^{2+} ions could, by itself, go some way (< 15%) to increasing activity, but not by nearly as much as observed.

We speculated (Hawkes et al., 1993) that the pH-dependence of k_{cat} might, in large part, be governed by the chemical step in the catalytic cycle. On the basis of structure-activity data for inhibition it was further suggested that, as part of this rate-determining step, the dianion of the phosphate group of enzyme-bound substrate might act as an internal base to remove the proton from C-2 of the glycerol side chain. In this case, the lower of the two $\text{p}K_a$ values observed would correspond to the apparent $\text{p}K_a$ governing the loss of a proton to give the phosphate dianion. Mn^{2+} might bind sufficiently close to directly lower this $\text{p}K_a$.

However, given that this would also reduce basicity, it is not then obvious how this might relate to the observed enhancement in rate.

Dependence of activity on a thiol

Historically, assays of IGPD have included high concentrations of thiol (0.14 M β -mercaptoethanol), since this was found to be a requirement from the first (Ames, 1957). Recently, this was reaffirmed for the wheat enzyme (Mano et al., 1993). In contrast, we found that 0.1 M β -mercaptoethanol had no influence on activity either with or without Mn^{2+} added to the assay or at either low (10 nM) or high (1 μM) concentrations of enzyme. Figure 5 shows the complete progress curve for conversion of IGP into IAP, both in the presence and in the absence of 0.1 M β -mercaptoethanol.

Comparing the amino acid sequences of IGPD derived from the DNA sequences of *S. cerevisiae* (Struhl, 1985), *Azospirillum brasilense* (Fani et al., 1989), *S. typhimurium* (Carlomagno et al., 1988), *Streptomyces coelicum* (Limauro et al., 1990), *Trichoderma harzianum* (Goldman et al., 1992) and *E. coli* (Chiariotti et al., 1986), there is no conserved cysteine residue. It seems, unlikely, therefore that a cysteine residue has a critical role in any evolutionarily conserved mechanism. Furthermore, non-redox active metals such as Cd^{2+} also assemble apo-IGPD into a catalytically active metalloenzyme (T. R. Hawkes and L. S. Edwards, unpublished work). Thus, whatever the need for thiol, it is more likely to represent a peculiarity of the enzyme from certain species and, perhaps, be involved in some facet of regulation rather than the fundamentals of catalysis.

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