An Unusual Hexose-ATP-Kinase with Two Catalytic Sites and a Role in Carbon Catabolite Repression in the Yeast *Schwanniomyces occidentalis*

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The hexose-ATP-kinase of wild-type *Schwanniomyces occidentalis* (CBS 819) is, like hexokinase PII of *Saccharomyces cerevisiae*, associated with carbon catabolite repression and phosphorylates D-glucose and D-fructose. The kinase of *Schw. occidentalis* repression-resistant (*Dog*^R) mutants phosphorylates D-glucose, but not D-fructose. Subjecting the wild-type enzyme to 45 °C for a few minutes appears to alter its activity, specificity and kinetic characteristics to that of the mutant enzyme. Fast protein liquid chromatography, with anion-exchange or gel-filtration columns, gel-electrophoresis, and DNA hybridization with the *hexI*⁺ mutant of the *HXK2* (hexokinase PII) gene of *Sacch. cerevisiae*, all failed to resolve more than one hexose-ATP-kinase in *Schw. occidentalis*. Hence *Schw. occidentalis* appears to have a single hexose-ATP-kinase, *M*_r ~72000, with two catalytic sites, analogous to the aspartate kinase/homoserine dehydrogenase of *Escherichia coli*. One site is hexokinase-like and associated with catabolite repression and the other is glucokinase-like. Values for apparent *K*_m were 7.2 mM-D-fructose and 0.55 mM-D-glucose ('hexokinase' site) and 0.078 mM-D-glucose ('glucokinase' site).

INTRODUCTION

In an industrial fermentation, D-glucose, liberated in, say, starch or sucrose hydrolysis, can slow down the whole process. This is because the repression of enzyme synthesis by glucose (carbon catabolite repression) is a major general regulatory system in yeasts (Mahler *et al.*, 1981). Many yeast enzymes are subject to glucose repression, such as those of (i) gluconeogenesis (Gancedo *et al.*, 1965; Gancedo & Schwerzmann, 1976; Haarasilta & Oura, 1975; Witt *et al.*, 1966), (ii) the tricarboxylic acid cycle (Polakis & Bartley, 1965), (iii) the glyoxylate cycle (Barnett & Kornberg, 1960), in addition to (iv) carriers and enzymes responsible for the transport and initial metabolism of exogenously supplied sugars and their derivatives (Barnett, 1976, 1981), and (v) amylases (McCann & Barnett, 1986).

Saccharomyces cerevisiae possesses at least three hexose-ATP-kinases, namely (a) two hexokinases (EC 2.7.1.1), PI and PII (Colowick, 1973), which phosphorylate both D-glucose and D-fructose and (b) the D-glucose-specific glucokinase (EC 2.7.1.12) (Maitra, 1970; Gancedo *et al.*, 1977; Maitra & Lobo, 1983). In addition to its catalytic role, hexokinase PII triggers glucose repression (Entian, 1980; Entian & Mecke, 1982). Repression-resistant mutants, selected in the presence of 2-deoxy-D-glucose (*Dog*^R mutants; see Zimmermann & Scheel, 1977), are deficient in PII. However, the relations between the hexose phosphorylating enzymes and their role in catabolite repression vary with different yeasts. For example, *Dog*^R

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Abbreviations: *Dog*^R, 2-deoxy-D-glucose resistant; FPLC, fast protein liquid chromatography; G/F ratio, ratio of rate of phosphorylation of D-glucose to that of D-fructose; PMSF, phenylmethylsulphonyl fluoride; TEA, triethanolamine.

mutants of *Rhodotorula glutinis* may lose activity of glucokinase, not hexokinase (Mahlberg *et al.*, 1985). Furthermore, from the work of McCann & Barnett (1984), *Schwanniomyces occidentalis* appears to have only a single hexokinase, which is lost in Dog^R mutants. The present paper reports some unusual characteristics of this enzyme.

METHODS

Organisms, growth and preparation of suspensions. Yeast stock cultures were maintained at 3 °C on slopes of Difco Bacto YM agar and all incubations were at 25 ± 0.5 °C. For enzymic studies, each culture (1 litre) of *Schwanniomyces occidentalis* Klöcker CBS 819 and its Dog^R mutant 819-11 (McCann & Barnett, 1984) was grown in a rotating (100–110 r.p.m.) 10 litre flask (Mitchell, 1949). The growth medium was Difco Bacto Yeast Nitrogen Base (without amino acids) (YNB) with 20 mM-D-glucose or D-xylose. A strain of *Saccharomyces cerevisiae* Meyen ex Hansen (catl.33-14A, obtained from K.-D. Entian) was grown similarly, but in YNB with 0.5% (w/w) Difco Bacto Yeast Extract and 25 mM-ethanol (as carbon source). When grown to about 0.4 mg dry weight ml⁻¹ (estimated from the optical density at 640 nm), the yeast was harvested by centrifugation for 10 min at about 5000 g and 4 °C and washed in ice-cold 50 mM-triethanolamine buffer (pH 7.6) (TEA), and the pellet was stored at -20 °C.

Enzyme preparations. Crude extracts were prepared (Ciriacy, 1976) by whirlimixing for 90 s about 70 mg dry wt of thawed yeast with 2.5 g fine glass beads (no. 9, Jencons) in 0.5 ml TEA, both ice-cold, in a plastic test tube (95 × 16 mm). A further 1.0 ml TEA, ice-cold, was mixed with the broken yeast and the suspension centrifuged at about 20000 g for 30 min at about 4 °C. The supernatant fluid was desalted through a Sephadex G25 column, giving a final protein concentration of between 200 and 500 µg ml⁻¹, and either purified or used directly for enzyme assays. Buffers contained 2 mM-phenylmethylsulphonyl fluoride (PMSF) (Fahrney & Gold, 1963; Schulze *et al.*, 1966) and 2 mM-DTT.

The hexose-ATP-kinase was purified from samples of extract (up to 4 ml) by fast protein liquid chromatography (FPLC; Pharmacia), using either an anion-exchange or a gel-filtration column, each at room temperature. The anion-exchange column, (Mono Q HR 5/5) was used with buffer A, 20 mM-piperazine (pH 6.0) or 20 mM-TEA (pH 7.6 or 8.6) and buffer B, composed of buffer A + 1.0 M-NaCl; the flow rate was 2 ml min⁻¹. Fractions of 0.2 ml were collected. Elution involved 4 ml buffer A and then a gradient of 20 ml buffer B, to give, finally, 0.35 M-NaCl. The gel filtration (Superose 12) column was used with 20 mM-TEA buffer (pH 7.6) and a flow rate of 0.5 ml min⁻¹. Fractions of 0.25 ml were collected. For some experiments, including the electrophoretograms, the purified samples were concentrated five- or tenfold by centrifugal ultrafiltration with Centricon Microconcentrators (Amicon).

Electrophoresis. Crude or purified extracts were electrophoresed on 94 × 76 mm cellulose acetate plates (Titan III, Helena Laboratories, Beaumont, Tx., USA) at 4 °C, using 10 mM-Tris/glycine buffer (pH 8.5) or 0.1 M-Tris/citrate (pH 7.6), as a continuous buffer system, for about 20 min at 200 V. These plates were designed for medical applications, but have been used effectively for separating dehydrogenases (e.g. Searle, 1985). Hexose-ATP-kinase activity was detected on the plates by overlaying, at about 20 °C for 15 min, with a solution of 100 mM-TEA (pH 7.6), gelled with 1.5% (w/v) agarose, containing 1.5 mM-NADP, 2 mM-ATP, 1 mM-D-glucose, 0.3 mM-phenazine methosulphate, 0.7 mM-(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl monotetrazolium bromide) (MTT), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (18 units ml⁻¹) and, when detecting phosphorylation of D-fructose, glucose-6-phosphate isomerase (EC 5.3.1.9) (20 units ml⁻¹).

Measurements of hexose phosphorylation. Phosphorylation of D-glucose or D-fructose, by crude or partly purified extracts, was estimated from the rate of the coupled NADP-linked oxidation of D-glucose 6-phosphate (Fromm & Zewe, 1962; Maitra, 1975), with increase in NADPH concentration measured at 340 nm. Reaction mixtures consisted of 1.0 mM-D-glucose or 20 mM-D-fructose, 1.0 mM-NADP, 1.0 mM-ATP and 10 units glucose-6-phosphate dehydrogenase ml⁻¹, with 5 units glucose-6-phosphate isomerase ml⁻¹ added for estimating D-fructose phosphorylation. In certain experiments, analogues replaced ATP, namely, CTP, ITP, GTP or UTP, each at 1.0 mM. Apparent K_m was estimated at least in triplicate, in each case, using the program Statstream (Bangham, 1985), running on an Apple II microcomputer. Values were chosen, by iteration, that best fitted the Michaelis-Menten equation to the experimental measurements (Colquhoun, 1971).

Plasmid. The structural gene for hexokinase PII is *HXK2* and its mutant allele is *hxx2* (Lobo & Maitra, 1977c), formerly called *HEX1* and *hex1*, respectively. The mutant alleles, affecting the regulatory but not the catalytic function of hexokinase PII, are designated *hex1'* (see Entian *et al.*, 1985). The YRp/*hex1'*-1804 plasmid was kindly supplied by K.-D. Entian.

Preparation of hybridization probe. The *hex1'* DNA fragment was purified from the YRp/*hex1'*-1804 plasmid. Caesium chloride-purified YRp/*hex1'*-1804 was digested with *Hind*III and *Pst*I (see Maniatis *et al.*, 1982) combined and run on a preparative agarose gel. The 0.87 kbp fragment, which contained most of the *hex1'* gene (Entian *et al.*, 1985), was recovered from the gel by the method of Dretzen *et al.* (1981) and used as the probe in hybridization experiments.

Fig. 1. Partial purification of hexose-ATP-kinase of *Schw. occidentalis* (CBS 819, wild-type) and *Sacch. cerevisiae* (cat1.33-14 A) by ion-exchange chromatography. For each yeast, crude extracts were applied to an anion-exchange (Mono Q) column, which was eluted with a concentration gradient of NaCl in 20 mM-piperazine buffer (pH 6.0). Phosphorylation of D-glucose was measured at 25 °C, as described in Methods. No activity was detected in fractions obtained with <0.13 M-NaCl. ●—●, CBS 819; ○—○, cat1.33-14 A; ■—■, NaCl.

Radioactive labelling of DNA. DNA fragments were labelled with [32 P]dCTP by nick translation (Kelly *et al.*, 1970).

Hybridization. After restriction digestion with (i) *Eco*RI and (ii) *Hind*III (see Maniatis *et al.*, 1982) and agarose gel electrophoresis, DNA fragments were adsorbed on to nitrocellulose filters and heated overnight at 65 °C. For hybridization with labelled DNA (Southern, 1975), the temperature was kept at 65 °C for 16 h. The hybridization filters were exposed to Fuji RX film (20 × 40 cm). In order to strengthen the radioactive signals, intensifying screens were used at -70 °C.

RESULTS AND DISCUSSION

Crude extracts of wild-type *Schw. occidentalis* CBS 819 phosphorylated both D-glucose and D-fructose, whilst corresponding rates of phosphorylation by extracts of the Dog^R mutant, 819-11, were only 20% that of the wild-type for D-glucose and 2% for D-fructose (McCann & Barnett, 1984). A naive interpretation of these results, in terms of the findings for *Sacch. cerevisiae* (Lobo & Maitra, 1977b; Entian, 1980), was that the wild-type possessed a glucokinase and a single hexokinase and that the latter was lost in the mutant. So, in order to test this interpretation, the wild-type was examined to establish the number and character of its hexose-ATP-kinases.

FPLC analysis

Attempts were made to separate the enzymes responsible for hexose-ATP-kinase activities by FPLC anion-exchange chromatography and by gel filtration. As a control, extracts of *Sacch. cerevisiae* were also chromatographed, as this yeast has already been shown to give three peaks of hexose-ATP-kinase activity (e.g. Barnard, 1975; Entian & Mecke, 1982; Fernandez *et al.*, 1985; Kopetzki & Entian, 1985). Although *Sacch. cerevisiae* did, indeed, give at least three peaks (Fig. 1), the elution of hexose-ATP-kinase from wild-type *Schw. occidentalis* CBS 819, with D-glucose as substrate, gave only a single activity peak, which also corresponded to the phosphorylative activity against D-fructose. Similarly, only a single peak was obtained with gel filtration. With *Schw. occidentalis*, the apparent M_r of the kinase was about 72 000, the same order of magnitude as hexokinase from *Sacch. cerevisiae* (about 100 000; Colowick, 1973). The hexose-ATP-kinase of the mutant 819-11 gave an identical peak.

Although in each experiment with the anion-exchange column, the peak for the *Schw. occidentalis* hexose-ATP-kinase was approximately symmetrical, the ratio of the rate of D-glucose phosphorylation to that of D-fructose phosphorylation (G/F) showed a slight increase in

Fig. 2. Attempted separation of hexose-ATP-kinase activities of *Schw. occidentalis*. Crude extracts of CBS 819 (wild-type) and 819-11 (mutant) were combined, so that each component gave about the same total activity of D-glucose phosphorylation. The mixed extracts were chromatographed on an anion-exchange column (Mono Q), eluted with a concentration gradient of NaCl in 20 mM-piperazine buffer (pH 6.0). Hexose phosphorylation was measured as described in Methods. ●—●, D-Fructose phosphorylation ('hexokinase' activity of enzyme 1); ○—○, D-glucose phosphorylation (combined 'hexokinase' activity and 'glucokinase' activities of enzymes 1 and 2); □—□, calculated 'glucokinase' activity of enzyme 2; ■---■, concentration of NaCl.

the last three fractions of the peak (0.6 ml in total); but, here, the activity was low and the experimental error correspondingly high. These observations were consistent with the presence in the wild-type of both hexokinase and glucokinase of the same molecular size and with only a slight difference in net charge. Assuming that there was loss of hexokinase (or hexokinase-like activity) in mutant 819-11, crude extracts of both wild-type and mutant were mixed, each with about the same activity towards D-glucose, and subjected to ion-exchange chromatography, in order to establish whether the two activities were separable.

Peaks were obtained for phosphorylation of D-fructose by enzyme from the wild-type and D-glucose by enzyme from both wild-type and mutant. Fig. 2 shows these peaks, the activity of each fraction being expressed as a percentage of that with maximum activity. Fractions 1–5 had a constant G/F ratio, indicating that a single enzyme (enzyme 1) is responsible for both phosphorylations. Fractions 6 to 14 gave a changing G/F ratio, providing evidence for the presence of a second (modified?) hexose-ATP-kinase (enzyme 2). From the results with the first five fractions, the G/F ratio was calculated for enzyme 1 and, from this, its rate of D-glucose phosphorylation calculated for the whole peak. By subtraction, the contribution of enzyme 2, presumably a modified form of enzyme 1, was also calculated and the relative position of the peak for its activity is also shown in Fig. 2.

Electrophoresis

The electrophoretogram bands for the hexose-ATP-kinase of crude or partly purified extracts of *Schw. occidentalis* wild-type and mutant 819-11 all had about the same mobility (Fig. 3). The distance moved towards the anode was the same for each preparation whether for D-glucose or D-fructose phosphorylation. Extracts of 819-11 gave no bands with D-fructose. By contrast, three separated groups of bands of D-glucose-phosphorylating activity were obtained with extracts of *Sacch. cerevisiae*, as described previously (e.g. Ramel *et al.*, 1971; Muratsubaki & Katsume, 1979). Multiple electrophoretic bands have been found for the hexokinase of other yeast species, such as *Hanseniaspora uvarum* (*Kloeckera brevis*) (Kopperschlager & Hofmann, 1969).

DNA hybridization

The gene that codes for hexokinase PI (*HXK1*) in *Sacch. cerevisiae* hybridizes strongly with that for hexokinase PII (*HXK2*) and its mutant allele, *hex1^r* (Entian *et al.*, 1984, 1985).

Fig. 3

Fig. 4

Fig. 3. Cellulose acetate plate electrophoresis of partly purified hexose-ATP-kinase of *Schw. occidentalis*. Movement in the gel was from top ($\triangleright$) to bottom ($\blacktriangleright$, anode). CBS 819 wild-type, incubated with D-glucose; 2, CBS 819 wild-type, incubated with D-fructose; 3, mutant 819-11, incubated with D-glucose.

Fig. 4. Southern blot of genomic DNA of *Schw. occidentalis* CBS 819 (wild-type) and of *Sacch. cerevisiae*, hybridized with a mutant hexokinase PII gene, *hexI*⁺. Lanes 1 and 2, *Sacch. cerevisiae* cat1.33-14 A DNA digested with *Eco*RI (lane 1), and with *Hind*III (lane 2); lanes 3 and 4, *Schw. occidentalis* DNA digested with *Eco*RI (lane 3) and with *Hind*III (lane 4). Digested DNA was run on agarose gel electrophoresis. The hybridization probe was a [³²P]dCTP-labelled *hexI*⁺ fragment.

With a ³²P-labelled *hexI*⁺ fragment as probe against *Sacch. cerevisiae* cat1.33-14 A, the *Eco*RI digest gave hybridization signals of 4.0 and 1.7 kbp and the *Hind*III-digest gave about 15, 11, 8.2 and 2.5 kbp, compared with a λ standard (Fig. 4). The results with *Eco*RI-restricted DNA agreed closely with results of K.-D. Entian (1986, personal communication), which gave signals of 4.0 for hexokinase PII and 1.65 kbp for hexokinase PI.

Using a ³²P-labelled *hexI*⁺ fragment as a probe against *Schw. occidentalis*, wild-type, the *Eco*RI digest gave a hybridization signal of 5.8 kbp and the *Hind*III-digest gave 6.4 kbp, compared with a λ standard (Fig. 4). Hence, there is a DNA sequence in *Schw. occidentalis* that shows homology with the *hexI*⁺ gene of *Sacch. cerevisiae*. In addition, only one signal was observed, which is evidence for the presence of only a single hexokinase gene in *Schw. occidentalis*.

Heat inactivation

About 99% of D-fructose phosphorylating ability was lost when partly purified *Schw. occidentalis* hexose-ATP-kinase was heated to 45 °C for 40 min, >96% being lost within 8 min. This was also found with crude extracts. There was a simultaneous loss of 73% of the ability to phosphorylate D-glucose, the residual 27% was stable at 45 °C for 400 min. During this time, another portion of the same enzyme preparation kept at 0 °C lost <20% phosphorylating

Table 1. Apparent Michaelis constants, K_m , for phosphorylation of D-fructose by the partly purified hexose-ATP-kinase of *Schw. occidentalis* CBS 819 and comparison with other yeasts

The values were obtained at pH 7.6 and 25 °C (see Methods).

	Expt. no....	Apparent K_m (mM)					
		D-Glucose			D-Fructose		
		1	2	3	1	2	3
<i>Schw. occidentalis</i>							
Wild-type ('hexokinase' + 'glucokinase')		0.36	0.35	0.32	7.2	7.5	6.9
Mutant 11 ('glucokinase')		0.091	0.077	0.065	—	—	—
Heated wild-type*		0.082	0.076	—	—	—	—
Calculated value for 'hexokinase' site		0.53	0.56	—	—	—	—
Values obtained for other species							
Hexokinases							
<i>Candida maltosa</i> †			0.2			—	
<i>Candida tropicalis</i> §			0.34			2.2	
<i>Rhodotorula glutinis</i>			0.1–0.2			2.0–3.3	
<i>Sacch. cerevisiae</i> ¶			0.1			0.7–1.2	
Glucokinases							
<i>Candida tropicalis</i> §			0.29			—	
<i>Rhodotorula glutinis</i>			0.10			—	
<i>Sacch. cerevisiae</i> ¶			0.02–0.4			—	

* Partly purified preparation heated to 45 °C for 30 min.

† Röber *et al.* (1984).

§ Hirai *et al.* (1977).

|| Mazón *et al.* (1975); Mahlberg *et al.* (1985).

¶ Slein *et al.* (1950); Sols *et al.* (1958); Maitra (1970, 1975); Lobo & Maitra (1977a); Fernández *et al.* (1985).

activity towards D-fructose and there was no detectable loss of activity towards D-glucose. Hence, of the total D-glucose phosphorylating activity, 73% was attributable to the heat-labile 'hexokinase' site and 27% to the heat-stable 'glucokinase' site.

When crude extract of wild-type *Schw. occidentalis* had been incubated at 45 °C for 30 min, the hexose-ATP-kinase gave a single peak when run through the gel-filtration column (Superose 12), coincident with the peaks for the enzymes from extracts of unheated wild-type and mutant.

Kinetic characteristics of the hexose-ATP-kinase

Table 1 shows apparent Michaelis constants, K_m , for D-glucose and for D-fructose, of the partly purified hexose-ATP-kinase of *Schw. occidentalis*, from the wild-type CBS 819, Dog^R mutant 819-11 and heat-treated wild-type; constants reported by other workers for different yeast species are also shown. Constants obtained with crude extracts were closely comparable to those with fractionated preparations.

The apparent K_m for D-glucose differed markedly between wild-type (mean 0.34 mM) and mutant (0.078 mM). This difference may be compared to that between the hexokinase and glucokinase of *Sacch. cerevisiae* (Table 1; Slein *et al.*, 1950; Sols *et al.*, 1958; Maitra, 1970). Extracts from wild-type *Schw. occidentalis*, maintained for 30 min at 45 °C, behaved indistinguishably from those of mutant 819-11. These results were consistent with the loss by the mutant of either (i) a hexokinase, leaving a glucokinase, or (ii) a catalytic site with hexokinase-like specificity, leaving a glucokinase-like site. Assuming one of these alternatives to be true, by subtracting individual measurements, a value was calculated for the apparent K_m for the *Schw. occidentalis* 'hexokinase' site (or hexokinase) as 0.55 mM-D-glucose.

The K_m of the hexose-ATP-kinase for D-fructose (7.2 mM) was much higher than that for D-glucose, as has been found for the hexokinases of other yeasts (Table 1). In aqueous solution at 25 °C, about 25% of D-fructose is in the furanose configuration (Horton & Wałaszek, 1982; Angyal, 1984). Assuming it to be the furanose and not the pyranose form of D-fructose that is the

substrate for these kinases (Slein *et al.*, 1950; Kalckar, 1985), the K_m for D-fructose would be about 1.8 mM.

The phosphorylation of D-glucose or D-fructose was measured with various nucleoside 5'-triphosphates other than ATP, namely CTP, ITP, GTP or UTP. Whilst CTP, GTP and UTP gave rates that were <1% that with ATP, ITP gave a significant rate of phosphorylation, about 6–7% of that with ATP, both with D-glucose or D-fructose for wild-type *Schw. occidentalis* and with D-glucose for mutant 819-11. That the ratios were the same for both D-glucose and D-fructose is consistent with a single enzyme being responsible for both phosphorylations. Marked activity with only ITP among the ATP analogues is consistent with findings for hexokinase in *Sacch. cerevisiae* (Purich *et al.*, 1973).

Conclusions

Two clear alternative interpretations of the results of these investigations are as follows. First, in *Schw. occidentalis* wild-type (CBS 819), a hexokinase and a glucokinase were separate enzymes, and the mutant 819-11 had lost the hexokinase. Alternatively, a single hexose-ATP-kinase of the wild-type had two catalytic sites, one hexokinase-like and the other glucokinase-like, the mutant having lost the hexokinase-like site.

Neither chromatography nor electrophoresis, nor yet DNA hybridization, gave any evidence of more than one hexose-ATP-kinase in either wild-type or mutant strains. However, there was slight separation of these two enzymes by anion-exchange, but not by gel-filtration chromatography. This separation could indicate that the modification to the enzyme in the mutant was also associated with a slight difference in charge or that adding the mutant enzyme to that of the wild-type made it possible to observe the second peak which, for the wild-type alone, was obscured by the large hexokinase peak. However, heating the wild-type enzyme appeared to alter its activity, specificity and kinetics, so that it was then closely comparable to the mutant enzyme. Hence, the authors tend to favour the view that there were two catalytic sites on a single polypeptide. This does not seem to have been reported previously for a hexose-ATP-kinase. However, such a 'double-headed' enzyme has been described for *Escherichia coli*, in which aspartate kinase (EC 2.7.2.4) and homoserine dehydrogenase (EC 1.1.1.3) have been shown to be located on one polypeptide, so that a single protein performs both functions (reviewed by Cohen & Dautry-Varsat, 1980).

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