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Purification and Characterization of Yeast Orotidine 5'-Monophosphate Decarboxylase Overexpressed from Plasmid PGU2*

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Orotidine 5'-monophosphate decarboxylase (ODCase) has been overexpressed in yeast 15C cells transformed with a plasmid carrying the *URA3* gene that encodes ODCase. Twenty g of cells having ODCase activity equal to 30 mg of pure enzyme per liter of cell culture were obtained after 9 h of galactose induction. To remove yeast proteases, a 60–90% ammonium sulfate fractionation step plus the addition of EDTA as an inhibitor of metallopeptidases was necessary. The purification protocol yielded ODCase that was protease-free and stable to storage at 4 °C for 16 months. The pure enzyme had a specific activity of 40 units/mg in 50 mM phosphate buffer, pH 6, and could be stored at −20 °C in 20% glycerol with retention of full activity for more than 2 years. The enzyme had a K_m for orotidine 5'-monophosphate of 0.7 μM at pH 6 and 25 °C. The molecular weight of the plasmid-derived ODCase monomer determined by electrophoresis on denaturing polyacrylamide gels was 29,500. ODCase sedimented through sucrose density gradients as a monomer of about 30 kDa at low protein concentration and in the absence of ligands that bind at the catalytic site. An increase in the sedimentation rate could be induced by increasing the ODCase concentration or by adding ligands that are competitive inhibitors. ODCase sedimented in a single band typical of a protein of 46 kDa at the highest protein concentration studied or in the presence of 50 mM phosphate or 933 μM substrate (orotidine 5'-monophosphate) or product (UMP). A dimer sedimenting as a protein of about 64 kDa occurred in the presence of 50 μM 6-azauridine 5'-monophosphate or 2 μM 1-(5'-phospho-β-D-ribofuranosyl) barbituric acid, competitive inhibitors of ODCase. These results resemble the ligand-induced subunit association of the ODCase domain of bifunctional UMP synthase and support the use of yeast ODCase as a model for ODCases from other species.

Conversion of orotidine 5'-monophosphate (OMP)[1] to uridine 5'-monophosphate (UMP), the last step in the *de novo* pyrimidine biosynthetic pathway, is catalyzed by orotidine 5'-monophosphate decarboxylase (ODCase, EC 4.1.1.23). In mammals ODCase is part of the bifunctional protein, UMP synthase, which also contains the preceeding enzymatic activity in the pathway, orotate phosphoribosyltransferase (1). The deduced amino acid sequence of yeast ODCase (2) shares 53–54% homology with the sequence of the ODCase domain of mouse (3) and human (4) UMP synthases, which are themselves 90% identical. In addition, the deduced amino acid sequences of ODCases from several other species share several well conserved regions with yeast ODCase (5).

In the 1970's ODCase was purified from commercial bakers' yeast by several groups (6–8). The amount of ODCase in crude yeast extracts is small, and therefore, large, bulky preparations were required to obtain pure protein. The purification described by Brody and Westheimer (6) yielded 15 mg of pure ODCase from 12 pounds of pressed bakers' yeast using a large (400 ml) affinity column and two additional column chromatography steps. Such laborious purification schemes to prepare ODCase from commercial yeast made obtaining the hundreds of milligrams of ODCase required for structural and mechanistic studies involving crystallography and NMR spectroscopy forbidding.

In 1987 Lue *et al.* (9) constructed a vector for overexpression of *URA3*, the gene which codes for yeast ODCase. This plasmid, pGU2, carries the *URA3* gene under control of the promoter for *GAL1*, along with a region of the 2-micron plasmid needed for maintenance of the plasmid at high copy number in yeast, and the *GAL4* gene which codes for the GAL4 protein (9). The GAL4 protein promotes transcription from the promoter for *GAL1* in response to galactose (10), thus allowing the expression of plasmid-derived proteins to be induced by adding galactose to the growth medium. Workers in Roger Kornberg's group (9) reported yields of 30 mg of ODCase per liter of yeast culture with a single purification step using yeast strain Sf657–2D transformed with plasmid pGU2.

We have utilized plasmid pGU2 to optimize expression of ODCase in a similar yeast strain, 15C, which was supplied to us by the Kornberg laboratory. Modifications to the published protocols for purification of yeast ODCase (6, 9) were essential in order to eliminate protease activities so that large amounts of highly pure, stable ODCase could be obtained.

Both the conserved amino acid sequence and the availability of large amounts of pure enzyme make yeast ODCase an ideal model for studying the structure and mechanism of ODCases. In reports from this laboratory, yeast ODCase purified by the protocol described herein has been crystallized for structural studies (11) and used in catalytic mechanism studies of the binding of a ^{13}C-labeled inhibitor using NMR spectroscopy (12), as well as in kinetic studies of ^{13}C isotope

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[1] The abbreviations used are: OMP, orotidine 5'-monophosphate; ODCase, orotidine 5'-monophosphate decarboxylase; BMP, 1-(5'-phospho-β-D-ribofuranosyl)barbituric acid; aza-UMP, 6-azauridine 5'-monophosphate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate, MES, 2(*N*-morpholino)ethanesulfonic acid.

effects (13). In this report we present details of the overexpression, purification, and characterization of plasmid-derived ODCase. Its structural and kinetic properties are compared with those reported for ODCase purified from commercial bakers' yeast. In addition, we examine the sedimentation of yeast ODCase in sucrose gradients and compare the effects of ligands in promoting different aggregation states of the enzyme with those observed for UMP synthase (14–16).

RESULTS AND DISCUSSION[2]

Overexpression of Yeast ODCase—We obtained plasmid pGU2 and yeast strain 15C from Andrew Buchman in Roger Kornberg's laboratory, who informed us that they no longer used strain Sf657–2D for overexpression of ODCase. Therefore, modifications to their published procedure for overexpression of yeast ODCase from strain Sf657–2D cells carrying the pGU2 plasmid (9) were necessary to obtain a high level of ODCase expression in yeast strain 15C. Using the published protocol (9), yeast 15C cells grew poorly in unsupplemented YP medium reaching an $OD_{660\,nm}$ of only 1.5 instead of 4 as was reported for Sf657–2D cells, and only a slight increase in the total ODCase activity occurred when cells grown in unsupplemented YP medium were harvested after 2 h of galactose induction (Table I).

Our improved protocol included addition of 2% sucrose to the YP medium for the initial growth of the cells and induction with galactose for 9 instead of 2 h. With these modifications yeast 15C cells grew to $OD_{660\,nm}$ of 2.53 in YP medium plus 2% sucrose prior to galactose induction, with a 2.6-fold increase in the cell wet weight when compared to growth in YP medium without sucrose (Table I). Harvesting cells after 9 h of galactose induction yielded an increase in the total ODCase activity of 4.4-fold in cells grown in YP medium without sucrose and 6.8-fold in cells grown in YP medium plus 2% sucrose, compared with the activity in cells grown in YP medium without sucrose which were harvested after only 2 h of galactose induction (Table I). The observed increase was due to an increase in both the total protein and the amount of ODCase. Our modified protocol typically yielded 18–22 g of cells (wet weight) and ODCase activity equal to about 30 mg of pure ODCase per liter of YP medium, as was reported for Sf657–2D cells transformed with plasmid pGU2 (9).

Purification and Stabilization of ODCase—We found that the ODCase protein overexpressed from plasmid pGU2, when purified by previous protocols (6, 9), was highly susceptible to proteolysis after storage for as little as 1 month at 4 °C (Fig. 1). These ODCase preparations contained high levels of protease activities, which completely destroyed the ODCase activity after 2 months of storage at 4 °C. Since we were interested in crystallizing the enzyme and conducting mechanism studies (both of which could require that the enzyme be kept for long periods at 4 °C or higher temperatures), it was necessary to stabilize the enzyme against proteolysis so as to maintain an active, homogeneous protein preparation.

In order to design a strategy for removing proteases, we first determined which of the three classes of yeast protease activities, distinguished by their pH optima (17, 18), were present at each step in the purification protocol. When PMSF, pepstatin A, and leupeptin were used as protease inhibitors as was done by Lue *et al.* (9), the cell lysate still contained significant amounts of basic and neutral protease activities. These two classes of protease activities include proteinase B and carboxypeptidase Y, both neutral proteases, as well as a number of metallopeptidases which are either neutral or slightly basic proteases (17). Yeast strain 15C contains a *pep4* mutation, which makes it deficient in both proteinase B (19) and carboxypeptidase Y (20). Therefore, we reasoned that metallopeptidases might comprise a significant portion of the residual protease activity in the enzyme preparations. Adding 2 mM EDTA in addition to the other protease inhibitors reduced the neutral protease activity measured in cell lysates by 70–75%. Including an ammonium sulfate precipitation step and collecting the 60–90% precipitated protein completely eliminated the remaining neutral and basic protease activities, as well as the small amount of residual acidic protease activity. The purified ODCase contained no measurable protease activity and was stable against proteolysis when stored for up to 16 months at 4 °C (*cf.* Fig. 2, *lane 12* with Fig. 1, *lane 4*). We have added 2 mM EDTA to the protease inhibitor mixture containing 1 mM PMSF, 1 μM pepstatin A, and 0.6 μM leupeptin and have routinely added this mixture to each buffer solution used during the ODCase purification.

Yeast ODCase overexpressed from plasmid pGU2 was purified to homogeneity in three purification steps (Table II and Fig. 2). The CM-52 cellulose column removed a small amount of contaminating proteins remaining after elution from the Affi-Gel Blue column (*cf.* Fig. 1, *lanes 1* and *2*). In a typical purification, about 60 mg of ODCase with a specific activity of 39–43 units/mg (at 25 °C in phosphate buffer, pH 6) or 70–80 units/mg (at 37 °C in Tris-HCl buffer, pH 7.4) was obtained from 4.8 liters of cell culture with a recovery of 30–40% (Table II). Recovery is dependent on the binding capacity of the Affi-Gel Blue column, which diminishes with repeated use. The specific activity at pH 6 is consistent with the 35–

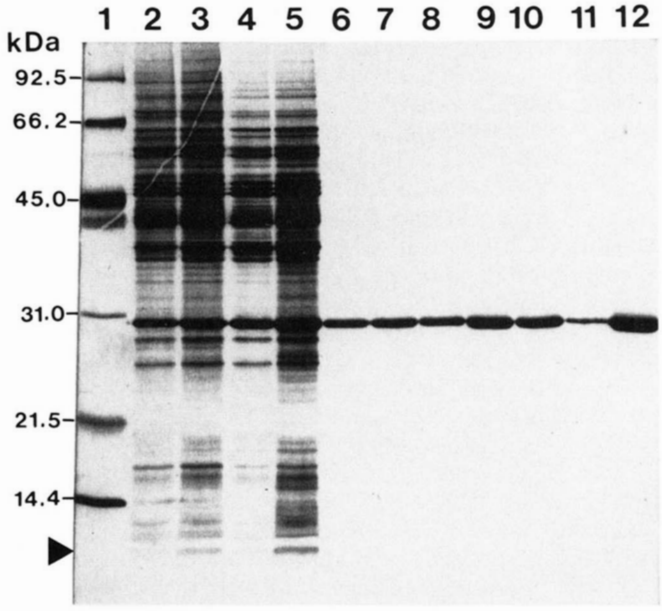

FIG. 2. **Purification of ODCase from yeast strain 15C cells transformed with pGU2.** Fractions from each purification step were run on a 12% SDS-polyacrylamide gel and silver-stained. The position of the dye front is indicated by the *arrowhead*. *Lane 1*, molecular weight markers; *lane 2*, clarified cell lysate (4 μg); *lane 3*, clarified cell lysate + 2 mM EDTA (4 μg); *lanes 4* and *5*, resuspended ammonium sulfate fraction (2.5 and 4 μg); *lanes 6* and *7*, Affi-Gel Blue column eluate (0.4 μg); *lane 8*, CM-52 cellulose column effluent (0.4 μg); *lanes 9–11*, YM10 concentrate (0.75, 0.5, and 0.25 μg); *lane 12*, pure ODCase stored for 16 months at 4 °C (1 μg).

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Tables I and II, Footnote 3, and Figs. 1 and 3–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

40 units/mg reported for other yeast purifications (6, 9). The pure ODCase monomer had a molecular mass of 29,500 daltons as estimated from its migration on a 12% SDS-polyacrylamide gel (Fig. 2, *lanes 9–12*). This value is close to the 29,000 daltons reported by Lue *et al.* (9) and is consistent with the value of 29,200 calculated for the amino acid sequence deduced from the nucleotide sequence of the *URA3* gene (2). This value is higher than the 27,500 reported by Brody and Westheimer (6) for ODCase purified from commercial bakers' yeast. While this difference may reflect a variation in the molecular weight standards used for estimating the weight of the ODCase monomer on SDS-polyacrylamide gels, we suspect that the difference could also be due to proteolysis of the enzyme prepared from autolyzed commercial bakers' yeast without the addition of protease inhibitors (6).

Subunit Association Studies—Previous studies of ODCase purified from commercial bakers' yeast have indicated that the enzyme exists in its native state as a simple dimer of 51 kDa (8). In contrast, a monomer and dimer form of the ODCase domain of mouse UMP synthase expressed in yeast cells have been reported (14). Studies of bifunctional UMP synthase isolated from mouse Ehrlich ascites cells have established that this UMP synthase can exist in three distinct conformational forms: a 3.6 S monomer, a 5.1 S simple dimer, and a 5.6 S dense dimer (15, 16). The aggregation states were identified by sedimentation of UMP synthase through sucrose gradients containing various ligands that bind to the enzyme. Data from these studies allowed Traut *et al.* (16) to suggest that a simple dimer, the 5.1 S species, is produced by ligand binding to the ODCase catalytic site and that formation of a dense dimer, the 5.6 S species, is promoted by effector binding to a noncatalytic, regulatory site.

In order to investigate the subunit association and aggregation state of yeast ODCase, the protein was subjected to centrifugation through 5–20% sucrose density gradients under a variety of experimental conditions. The effects of phosphate and enzyme concentration on the sedimentation of yeast ODCase were first examined in order to determine optimal conditions for studying the effect of nucleotide ligands on the subunit association of ODCase. We observed that ODCase stored in 50 mM sodium phosphate buffer migrated through sucrose gradients at a molecular mass intermediate to that expected for a monomer (29.5 kDa) or a dimer (59 kDa). A similar effect of orthophosphate on the sedimentation of UMP synthase has been attributed to the rapid equilibration of the monomer and dimer forms of the enzyme (15). When protein from an ammonium sulfate precipitation step (ODCase specific activity = 15 units/mg) was loaded onto a sucrose gradient in the amount of 10, 50, or 200 μg (equivalent to about 2, 10, and 40 μg of pure ODCase), a single ODCase activity peak was observed in each gradient. The apparent molecular masses of ODCase in gradients loaded with the three protein concentrations were 29, 36, and 46 kDa, respectively. We attribute this change in apparent mass to an equilibrium between the monomer and dimer forms of ODCase induced by a mass action effect as the ODCase concentration is increased. In order to eliminate the effects that protein concentration and phosphate have on the aggregation and subunit association of yeast ODCase, sucrose gradients to determine the effect of nucleotide ligands were run using a small amount of pure ODCase that had been dialyzed against Tris buffer.

When pure ODCase (2.2 μg) was sedimented through a 5–20% sucrose gradient in the absence of any nucleotide, ODCase migrated as a monomer of about 30 kDa (Fig. 3). Under identical conditions, ODCase sedimented as a dimer of about 64 kDa in a gradient containing either 50 μM aza-UMP or 2 μM BMP (Fig. 3). Gradients containing either OMP or UMP at a concentration of 933 μM both yielded an ODCase activity peak at a position in the gradient corresponding to a molecular mass of 46 kDa, which is intermediate between the monomer and dimer (Fig. 3). Since the ODCase activity loaded onto the gradient was sufficient to consume all of the OMP present during the course of the 40-h centrifugation, this intermediate species probably resulted from binding of UMP to ODCase in both cases.

In summary, changes in the aggregation and/or subunit association state of yeast ODCase can be influenced by protein concentration, phosphate, and nucleotide ligands. Two distinct species of the yeast ODCase were identified: a 30-kDa monomer observed at low enzyme concentration in the absence of phosphate and other ligands, and a 64-kDa dimer observed in the presence of aza-UMP or BMP.

Based on gel filtration and sucrose density gradient studies, the native state of ODCase purified from commercial bakers' yeast was considered to be a dimer of 51 kDa (8). The fact that a monomer of the yeast ODCase in solution had not been observed previously can be explained in light of our results, which suggest that the 51-kDa species of ODCase probably corresponds to the 46-kDa intermediate observed in the presence of UMP, phosphate, or at high protein concentration. The previous protocol (8) used phosphate buffer during the purification and sucrose density gradient centrifugation, which can cause ODCase to migrate at a higher apparent molecular mass. Also, the protein concentrations used during sucrose gradient centrifugation and gel exclusion chromatography could have been sufficient to promote subunit association. In each of the subsequent purifications of yeast ODCase (6, 9, 21) in which aza-UMP was used to elute the enzyme from affinity columns, no attempt was made to remove residual aza-UMP from the enzyme. As we have shown, a substantial amount of the inhibitor remains bound to the enzyme and must be removed by sequential dialysis (see Fig. 4 and "Results" in Miniprint). Therefore, in these cases the residual bound aza-UMP may have been sufficient to promote subunit association of the enzyme and preclude observation of the monomer form of ODCase.

Changes in the subunit association of yeast ODCase in the presence of ligands that bind to the catalytic site of the enzyme are very similar to those reported for the ODCase domain (14) and the intact UMP synthase (15, 16) from mouse. Such changes in the aggregation state of multisubunit enzymes have been suggested as a means of regulating enzymatic activity (22). Indeed, Traut *et al.* (16) have hypothesized that the 5.6 S dense dimer is promoted by effector binding to a regulatory site and is the only form which has ODCase activity. While we have observed both a monomer and a dimer form of yeast ODCase, we have no evidence for a species equivalent to the dense dimer species observed in UMP synthase. In this respect, yeast ODCase more closely resembles the isolated ODCase domain of the bifunctional mouse UMP synthase.

Results in this report have shown that properties of the plasmid-derived ODCase are very similar to those of the enzyme prepared from commercial bakers' yeast. In addition, our results have shown that yeast ODCase closely resembles the ODCase domain of UMP synthase in its subunit association properties, thus adding to kinetic and sequence data which support its use as a model for ODCases. Therefore, cell extracts containing the plasmid-derived yeast ODCase are excellent sources for obtaining large quantities of pure enzyme for studies on the structure and mechanism of ODCases.

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Supplemental Material to **Purification and Characterization of Yeast Orotidine 5'-monophosphate Decarboxylase Overexpressed from Plasmid pGU2**

by Juliette B. Bell and Mary Ellen Jones

EXPERIMENTAL PROCEDURES

MATERIALS. [7-^{14}C]-OMP and [methyl-^{14}C]methylated-methemoglobin were obtained from DuPont-New England Nuclear. OMP, 6-azauridine 5'-monophosphate (aza-UMP), 1-(5'-phospho-β-D-ribofuranosyl) barbituric acid (BMP), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, and 2[N-morpholino]ethanesulfonic acid (MES) were purchased from Sigma. [^{3}H]aza-UMP was synthesized by Moravek Biochemicals, Inc., Brea, CA. Yeast extract, bacto-peptone, bacto-agar, and yeast nitrogen base without amino acids were from Difco. Affi-Gel Blue was from Bio-Rad and CM-52 cellulose was from Whatman. Yeast plasmid pGU2 and yeast strain 15C (*ade2*-3,112 *ura*3-52 *his4*-580 *trp*1Δ *pep*4-3) were gifts from Drs. Andrew R. Buchman and Roger Kornberg, Department of Structural Biology, Stanford University School of Medicine, Stanford, CA. Other materials were of the highest grade commercially available.

OVEREXPRESSION OF ODCase. ODCase was overexpressed by modifications to the method of Lue et al. (9) as follows. Yeast strain 15C cells were transformed with plasmid pGU2 DNA by a standard yeast transformation procedure (23). Transformants were selected by their ability to grow in the absence of leucine. Colonies appeared in 4-5 days and were picked and grown at 30° C for 16 h (OD_{660} = 1.2-1.6) in 10 ml of synthetic complete minimal (SCM) medium containing 2% dextrose. SCM medium consisted of 0.67% bacto-yeast nitrogen base without amino acids and 0.8 g/l of a mixture containing: 4% adenine, tryptophan, histidine, arginine, and methionine; 6% tyrosine, lysine, and isoleucine; 10% phenylalanine and valine; and 40% threonine. To check for the ability of cells from each colony to achieve high expression of ODCase activity, 1 ml of the culture was diluted into 9 ml of SCM medium containing 2% sucrose and 2% galactose and grown for 16 h at 30° C. Five ml of cells from each culture was spun for 5 min at 3,000 x g and the cells were broken by vortexing for 2 min with 1 g of 0.5 mm glass beads in 0.5 ml lysis buffer consisting of 50 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, 2 μM pepstatin A, 0.6 μM leupeptin, and 10% glycerol. The ODCase activity was measured, and cell cultures which demonstrated high levels of ODCase activity (8-10 units per ml of lysate) were combined. The cell density was estimated by measuring the OD_{660}, and sufficient cells were added to 250 ml of SCM medium containing 2% sucrose to give 4 x 10^6 cells/ml. The preculture was grown at 30° C with vigorous shaking (250 rpm) for 16 h (OD_{660} = 1.3-1.6) and then diluted to 2 x 10^6 cell/ml into 600 ml of YP medium (1% bacto-yeast extract, 2% bacto-peptone) containing 2% sucrose. These cells were grown at 30° C with vigorous shaking in 2 liter flasks for 20 h (OD_{660} = 2.3-2.5) and then induced by adding galactose to 2% final concentration. After an additional 9 h of growth, the cells were harvested. This growth protocol typically yielded a cell wet weight of 18-22 grams per liter of YP medium.

PURIFICATION OF YEAST ODCASE. ODCase was purified by modification of published methods (6, 9) as follows. Yeast cells grown as described above were harvested by centrifugation at 6000 x g for 10 min. All operations were carried out at 4° C. Cells were resuspended in lysis buffer to wash the cells and recentrifuged in preweighed centrifuge tubes. The cell pellet was weighed and the cells suspended in 2 ml of lysis buffer/gm cells. Cells were mixed with an equal volume of 0.5 mm glass beads and broken using four 60 s pulses of a Bead Beater (Biospecs Products, Bartlesville, OK) with 60 s intervals for cooling between pulses. Cell debris was removed by centrifugation at 14,000 x g for 20 min and the supernatant was spun for 3 h at 100,000 x g. The protein was fractionated using solid ammonium sulfate and the 60-90% precipitate was resuspended in 60 ml of lysis buffer. Ammonium sulfate was removed by dialyzing against 1 liter of lysis buffer (minus pepstatin A and leupeptin) for 3 h with a buffer change each h. Following dialysis, pepstatin A and leupeptin were added to the protein solution to 1 μM and 0.6 μM, respectively. Affinity chromatography on an Affi-Gel Blue column (4.4 x 4 cm) was as previously described (9) except that 2 mM EDTA and 10% glycerol were added to the buffer. ODCase was eluted from the column with the same buffer containing 50 μM aza-UMP. The combined fractions were dialyzed against 1.5 liters of 5 mM sodium phosphate buffer, pH 6.0, which contained 5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, and 10% glycerol for 90 min with a buffer change after 45 min. Following dialysis, pepstatin A, leupeptin, and aza-UMP were added to the protein solution to 1 μM, 0.6 μM, and 100 μM, respectively. This protein solution was passed over a CM-52 cellulose column [2.2 x 40 cm, (6)] which had been previously equilibrated with the above 5 mM sodium phosphate buffer containing aza-UMP, pepstatin A, and leupeptin in addition to the other components. The effluent containing ODCase activity was concentrated to one-tenth volume using an Amicon Stirred Cell ultrafiltration apparatus and YM-10 filter (Amicon Division, W. R. Grace & Co., Danvers, MA).

Following the purification, bound aza-UMP was removed from the enzyme by sequential dialysis (24) against the following buffers in 100-fold excess over the enzyme volume. Buffer A containing 300 mM sodium phosphate, pH 6.0, 5 mM 2-mercapto-ethanol, 10% glycerol, and 10 mM UMP was used for 72 h with the buffer being changed at 5, 10, 26, 34, 49, and 57 h; Buffer B containing 300 mM sodium phosphate, pH 6.0, 5 mM mercapotoethanol, and 10% glycerol was used for 48 h with buffer changes at 9, 24, and 36 h; and Buffer C containing 50 mM sodium phosphate, pH 6.0, 5 mM 2-mercaptoethanol, 20% glycerol, 1 mM PMSF, and 2 mM EDTA was used for 24 h with a buffer change at 8 h. Following dialysis, pepstatin A and leupeptin were added to the protein solution to 1 μM and 0.6 μM, respectively. The pure enzyme was stored in this buffer at -20° C. Enzyme used for sucrose gradient experiments was further dialyzed to remove phosphate by substituting 20 mM Tris-HCl, pH 7.5 (4° C) for the sodium phosphate used in dialysis Buffer C.

ENZYME ASSAYS. ODCase activity was measured by the release of [^{14}C]CO_2 from [7-^{14}C]OMP quantitated as previous described (25) at 37° C in assay mixtures containing 20 mM Tris-HCl (pH 7.4 at 37° C), 0.1 mM EDTA, and 40 μM [7-^{14}C]OMP, or at 25° C in 50 mM sodium phosphate buffer, pH 6.0, containing 5 mM 2-mercaptoethanol and 50 μM [7-^{14}C]OMP. Kinetic assay mixtures containing 7.7 x 10^{-11} M ODCase and the indicated concentrations of OMP were incubated for 1 min at either 25° C or 37° C in the buffer stated in the figure legend. For all kinetic assays, 5 percent or less of the substrate was consumed during the reaction. The specific activity of ODCase is reported as units/mg protein, where a unit of ODCase activity is defined as 1 μmole of OMP converted to UMP and CO_2 per min.

PROTEIN DETERMINATION. Protein was determined by the dye binding method of Bradford (26), using bovine serum albumin as a standard.

SUCROSE DENSITY GRADIENT CENTRIFUGATION. Linear sucrose density gradients of 5-20% (w/v) were prepared using a 10 ml gradient maker (MRA, Clearwater, FL). Each gradient had a final volume of 5.2 ml and was prepared by adding 2.6 ml of 5% sucrose to the mixing chamber and 2.6 ml of 20% sucrose to the reserve chamber of the gradient maker. The gradient was pumped into the bottom of a Beckman Ultra Clear centrifuge tube (1/2 x 2 in) at 2 ml/min using a peristaltic pump. Sucrose solutions were prepared in 20 mM Tris-HCl, pH 7.5 (4° C) containing 10% glycerol, 5 mM 2-mercaptoethanol, and nucleotides as indicated. Prepared gradients were chilled at 4° C for 1 h prior to loading with protein sample. Protein samples were prepared in a volume of 10 μl in the same Tris buffer without glycerol. Each sample contained an ODCase protein preparation as indicated in the text and the following molecular weight standards (12 μg each): lysozyme (14,400), carbonic anhydrase (31,000), bovine serum albumin (66,200), and yeast alcohol dehydrogenase (80,000). Gradients were centrifuged at 4° C for 40 h at 228,000 x g using a Beckman SW65 rotor and L5-75 ultracentrifuge. Gradients were fractionated into 0.1 ml fractions using an Isco Model 640 density gradient fractionator at a flow rate of 0.5 ml/min. The positions of the marker proteins in the gradient were estimated by a qualitative analysis of SDS-polyacrylamide gels containing fractions from the gradients and the standard proteins. ODCase was determined by enzyme assay.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS. SDS-polyacrylamide gel electrophoresis was carried out with 12% slab gels using the discontinuous buffer system of Laemmli (27). SDS-PAGE low molecular weight standards, obtained from Bio-Rad, were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). Gels were silver stained as previously described (28).

PROTEASE ASSAYS. Protease activity was measured by modifications to the procedure of Anson (29) using [methyl-^{14}C]methemoglobin as described by New England Nuclear[3]. Three classes of proteases were assayed individually using substrate solutions containing 0.2 μCi of [methyl-^{14}C]methemoglobin (2 mg bovine hemoglobin and 0.2 μCi [methyl-^{14}C]methemoglobin, final specific activity = 0.1 μCi/mg) in one of the following buffer solutions: acidic protease assay- 0.2 ml of 0.1 mM sodium citrate, pH 3.1, 0.05 ml of 0.3 N HCl, and H_2O to 1 ml; neutral protease assay- 0.1 ml of 1 M Tris-HCl, pH 7.5, and H_2O to 1 ml; basic protease assay- 0.1 ml of 1 M Tris-HCl, pH 8.5, 0.48 g urea, and H_2O to 1 ml. Substrate solutions and ODCase samples of 50 μl containing 0.15-0.25 mg of protein were preincubated separately at 39° C for 20 min. For each substrate solution, 50 μl was added to each ODCase sample and the mixtures were incubated at 39° C for 2 hr. The reactions were stopped by adding 100 μl of 10% trichloroacetic acid to each sample. Precipitates were pelleted in an Eppendorf microfuge for 5 min. The supernatants were removed and 175 μl of each was counted in 10 ml of Safety-Solve liquid scintillation fluid (Research Products International, Mount Prospect, IL). The number of solubilized μg of hemoglobin (Hb) was calculated from the number of cpm and the specific activity of the methemoglobin used. Protease activity is reported as μg Hb/mg total protein in the ODCase sample being tested.

[3] Technical bulletin: "New Sensitive Assay for Proteolytic Enzymes", New England Nuclear, 549 Albany Street, Boston, MA 02118.

TABLE I

Galactose induction of ODCase in yeast 15C cells

Cells were grown at 30° C in either YP[a] medium or YP medium plus 2% sucrose. Galactose was added immediately after the 0-hrs time point samples were removed. The other samples were taken at the indicated times. All samples consisted of 5 ml of the appropriate cell culture and were removed sterilely and processed as described under **EXPERIMENTAL PROCEDURES** - "Overexpression of ODCase".

Time	OD_{660}	Cell Wt.	Protein	Sp. Act.	Yield	
hrs		*g/l*	*mg/ml*	*units/mg*	*mg*	*units*
		YP Medium Without Sucrose				
0	1.54	5	1.2	4.8	0.6	2.9
2	1.60	6	1.6	4.4	0.8	3.6
4	1.72	8	1.7	4.6	0.9	4.0
6	1.90	9	2.8	4.1	1.4	5.8
9	2.28	13	5.6	5.6	2.8	15.8
10	2.40	13	6.3	5.4	3.2	16.9
12	2.50	16	7.6	3.1	3.8	12.0
		YP Medium Plus 2% Sucrose				
0	2.53	13	7.1	3.8	3.6	13.4
2	2.54	14	6.3	4.9	3.2	15.5
4	2.62	14	6.4	5.8	3.2	18.5
6	2.73	17	6.8	N.D.[b]	3.4	N.D.
9	2.89	20	8.4	5.8	4.2	24.5
10	2.91	21	6.5	6.2	3.3	20.3
12	2.94	22	6.5	6.5	3.3	21.2

[a]YP medium = 1% bacto-yeast extract, 2% bacto-peptone.

[b]N.D. = not determined.

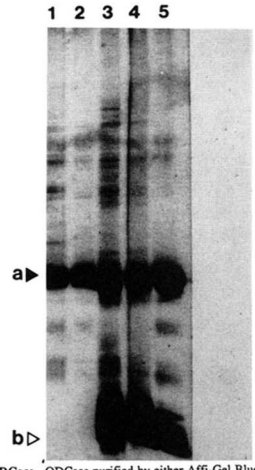

FIG. 1. **Proteolysis of yeast ODCase.** ODCase purified by either Affi-Gel Blue chromatography only (Lanes 1 & 3) or by Affi-Gel Blue and CM-52 cellulose chromatography (Lanes 2, 4 & 5) was run on a 12% SDS-polyacrylamide gel and silver stained. Neither protocol included an ammonium sulfate step or the presence of EDTA in the buffers. The positions of the ODCase monomer (**a**) and the dye front (**b**) are indicated. Each lane contains 6 µg of ODCase stored at 4° C for the indicated times: Lanes 1 & 2 - 1 week; Lanes 3 & 4 - 8 weeks; and Lane 5 - 4 weeks.

TABLE II

*Purification of yeast ODCase overexpressed from yeast 15C cells carrying plasmid pGU2**

Cells (105 g) from 4.8 liters of YP medium plus 2% sucrose were harvested after 9 h of galactose induction and processed as described in the text.

Fraction	Specific Activity	Total Volume	Total Protein	Total Activity	Percent Yield[a]	Purification Fold
	units/mg	*ml*	*mg*	*units*		
Clarified Lysate	6.2	193	2200	13640	100	1.0
Ammonium Sulfate	9.5	66	1030	9785	72	1.5
Affi-Gel Blue	76.2	77	72.4	5517	40[b]	12.3
CM-52 Cellulose	102.3[c]	85	65.5	5517	49	16.5
YM10 Concentrate	78.3	9	53.1	4158	30	12.6
Dialyzed ODCase	75.8	9	53.0	4025	30	12.2

*Enzyme activity was measured at 37° C in Tris-HCl at pH 7.4.

[a]Percent yield is based on the ODCase activity recovered.

[b]Thirty-nine percent of the ODCase activity of the ammonium sulfate fraction loaded was not retained on the Affi-Gel Blue column.

[c]The CM-52 cellulose fractions contain 100 µM aza-UMP which stabilizes ODCase against loss of activity from dilution.

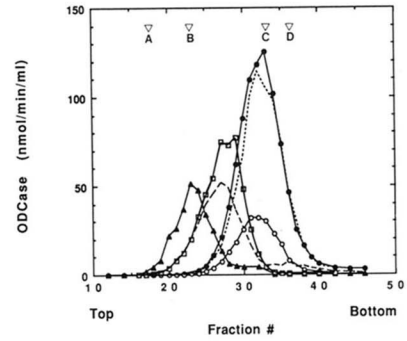

FIG. 3. **Effect of ligands on sedimentation of ODCase through sucrose gradients.** Protein solutions containing 2.2 µg of pure, dialyzed ODCase were loaded onto individual 5-20% sucrose gradients containing the indicated ligand and centrifuged for 40 h. The position of ODCase in the gradient was determined by enzyme assay. Molecular weight standards were: A - lysozyme (14,400); B - carbonic anhydrase (31,000); C - bovine serum albumin (66,200); and D - yeast alcohol dehydrogenase (80,000). Gradients containing either 50 µM aza-UMP (●), no ligand (▲), or 933 µM OMP (– –) were run in one experiment. Gradients containing 50 µM aza-UMP (----), 2 µM BMP (○), or 933 µM UMP (□) were run in a separate experiment. Values for the ODCase activity in gradients containing no ligand or BMP are multiplied by a factor of 10.

RESULTS

SEQUENTIAL DIALYSIS. Following purification, ODCase was subjected to sequential dialysis to remove residual bound aza-UMP. Using [^{3}H]aza-UMP, we determined that 0.65 mole of aza-UMP remains bound per mole of ODCase dimer following dialysis for 2 h against 500 X volume of 50 mM sodium phosphate, pH 6, containing 5 mM 2-mercaptoethanol and 10% glycerol with a buffer change after 1 h. Extensive dialysis for 72 h against a buffer containing 300 mM phosphate and 10 mM UMP (24) was required to remove the bound aza-UMP (Fig. 4). Analysis of a difference UV scan of dialyzed and undialyzed ODCase in the region of 240 to 280 nm showed an absorbance at the λ_{max} for aza-UMP (262 nm) corresponding to about 0.7 mole of aza-UMP per mole of ODCase dimer in the undialyzed sample (data not shown). In previous purifications of yeast ODCase (6, 9, 21), no attempts were made to remove residual aza-UMP. Although the bound aza-UMP apparently does not interfere with measurement of ODCase activity (presumably because it stabilizes the enzyme against dilution and has a K_i value approximately equal to the K_m value for OMP), we found that a ligand-free enzyme preparation was necessary for subunit association studies.

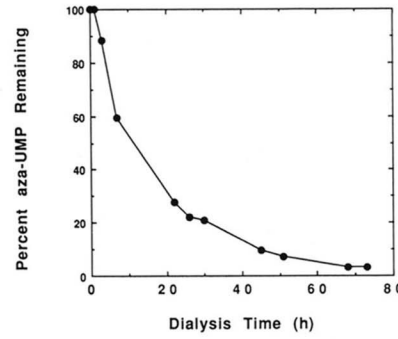

FIG. 4. **Removal of residual aza-UMP from ODCase by dialysis.** ODCase (65 µM dimer) and [^{3}H]aza-UMP (65 µM) in 1.8 ml were mixed overnight at 4° C. Unbound aza-UMP was removed by dialysis against 1 liter of 50 mM sodium phosphate buffer, pH 6, containing 5 mM 2-mercaptoethanol and 10% glycerol for 2 h with a buffer change after 1 h. Two 5 µl aliquots of the protein solution were removed and counted in 5 ml of Safety-Solve liquid scintillation fluid. The amount of aza-UMP measured (42 µM or 0.65 mole aza-UMP per mole of enzyme) was taken as the amount bound to the protein and is indicated as 100% aza-UMP remaining in the graph. The dialysis buffer was changed to 300 mM sodium phosphate, pH 6 containing 5 mM 2-mercaptoethanol, 10 mM UMP, and 10% glycerol. Dialysis was continued for 72 h with aliquots removed and counted at the time points indicated. Each point is the average of three determinations.

GLYCEROL STABILIZATION. Pure ODCase is totally inactivated by freezing (8), therefore, we sought to stabilize the enzyme by including glycerol in the storage buffer (24). As shown in Fig 5, the enzyme activity was stabilized for storage at -20° C for more than 2 years by including as little as 10% glycerol. Our purified ODCase is routinely stored at -20° C in a buffer solution containing 50 mM sodium phosphate, 5 mM 2-mercaptoethanol, 20% glycerol, 2 mM EDTA, 1 mM PMSF, 1 µM pepstatin A, and 0.6 µM leupeptin. We also found that 10% glycerol added to buffer solutions used during purification steps stabilized the enzyme to loss of activity from dialysis or dilution. Ten percent glycerol was routinely added to all buffers and did not interfere with binding of ODCase to the Affi-Gel Blue resin or at any other purification step.

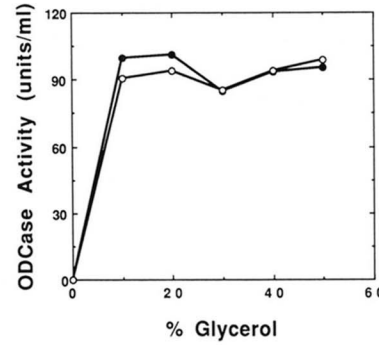

FIG. 5. **Glycerol stabilization of ODCase.** Purified ODCase was stored at -20° C in sodium phosphate buffer, pH 6, containing 50 mM sodium phosphate, 5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, 1 µM pepstatin A, 0.6 µM leupeptin, and the indicated concentration of glycerol. After 4 months (open circles) and 27 months (closed circles) aliquots were removed from each sample and assayed for ODCase activity.

KINETIC STUDIES. Several conflicting K_m values for OMP have been reported for ODCase purified from commercial baker's yeast. Brody and Westheimer (6) reported a K_m for pure ODCase of 1.5 μM OMP measured at 25° C in 50 mM sodium phosphate, pH 6.0, and 5 mM 2-mercaptoethanol. Under the same experimental conditions, Umezu et al. (8) reported a K_m of 5.0 μM OMP. At 37° C in Tris-HCl buffer, pH 7.4, and 1 mM dithiothreitol, Fyfe et al. (7) reported two K_m values for yeast ODCase observed at low or high OMP concentrations: a K_m of 0.45 μM when OMP was below 3-7 μM, and a K_m of 2.4 μM when OMP was above 7-10 μM. Brody and Westheimer (6) reported only one K_m value of 0.5 μM under similar experimental conditions with OMP concentrations ranging from 1 to 7 μM.

Kinetic parameters for the plasmid-derived ODCase were examined in order to compare the values obtained with values reported for the enzyme purified from commercial baker's yeast. At 25° C in 50 mM sodium phosphate, pH 6.0, and 5 mM 2-mercaptoethanol, we obtained a K_m of 2.4 μM with OMP concentrations ranging from 0.25 to 4 μM. Under the same experimental conditions using MES buffer instead of phosphate, the K_m was 0.7 μM. The V_{max} for OMP decarboxylation in both buffers was the same (0.065 nmol/min), and a double-reciprocal plot of the two substrate saturation curves showed competitive inhibition by orthophosphate (Fig. 6). Therefore, the K_m for OMP in the absence of inhibitors is 0.7 μM. These data confirm that phosphate is a competitive inhibitor of ODCase, and the K_i of 18.5 mM for phosphate calculated from these data is consistent with an independently determined value of 20 mM (30). Previously determined values for the K_m of ODCase in phosphate buffer (6, 8) are therefore higher than the true value due to inhibition by phosphate. At 37° C in 50 mM Tris-HCl, pH 7.4, a K_m of 2.6 μM was calculated over the OMP range of 0.25 to 10 μM. This value is consistent with the K_m value obtained at high OMP concentrations by Fyfe et al. (7), however, we did not observe two K_m values under these conditions.

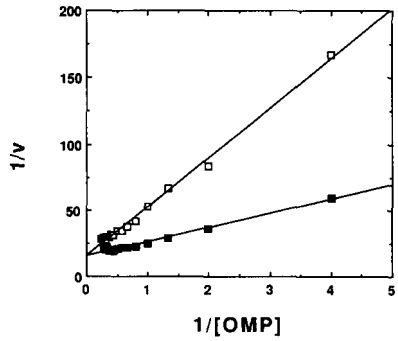

FIG. 6. **Competitive inhibition of ODCase by phosphate.** Each assay contained 50 mM of the indicated buffer at pH 6, 5 mM 2-mercaptoethanol, 7.7×10^{-11} M ODCase, and 0.25-4.0 μM OMP in a final volume of 0.5 ml. Assays were for 1 min at 25° C. Each point on the curve represents an average of 6 determinations: open squares - sodium phosphate buffer; closed squares - MES buffer.