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 metalloproteases was necessary. The purification protocol yielded ODCase that was protease-free and stable at 4 °C for storage at −20 °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 46 kDa at low protein concentration and in the absence of ligands that bind to 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) 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 preceding 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 13C-labeled inhibitor using NMR spectroscopy (12), as well as in kinetic studies of 13C isotope...
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 (14–15).

RESULTS AND DISCUSSION

Overexpression of Yeast ODCase—We obtained plasmid pGU2 and yeast strain 15C from Andrew Buchanan 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 OD₆₆₆ₘₙₙ 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 OD₆₆₆ₘₙₙ 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-
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40 units/mg reported for other yeast preparations (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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EXPERIMENTAL PROCEDURES

MATERIALS: 1-14C-OMP (amu666) and [3H]uridine were obtained from DuPont-New England Nuclear (Boston, MA). DMSO, 5-azacytosine, and cytosine arabinoside were purchased from Sigma. [14C]Uridine, [14C]thymidine, and [14C]adenosine were purchased from DuPont-New England Nuclear (Boston, MA). All other reagents were purchased from standard commercial sources.

OVEREXPRESSION OF ODCase. ODCase was overexpressed in yeast as described in the text. The overexpressed ODCase was purified to apparent homogeneity by standard chromatographic procedures. The purity of the purified ODCase was determined by SDS-PAGE and protein-sepharose affinity chromatography (Bio-Rad, Hercules, CA). The purified ODCase was used in all experiments.

PROTEIN DETERMINATION. Protein was determined by the method of Bradford (26) or by the dye-binding method of Bradford (27). Protein concentrations were determined by the method of Bradford (26) using BSA as a standard.

PURIFICATION OF YEAST ODCASE. ODCase was purified by modification of published methods. In brief, yeast cells grown in the presence of 5-azacytosine and 5-azacytosine were harvested by centrifugation at 3,000 x g. Aliquots were used for in vivo and in vitro experiments as described in the text. The in vivo experiments were performed with yeast cells that had been treated with 5-azacytosine and 5-azacytosine. The purified ODCase was used in all experiments.

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TABLE I

| Time | OD₅₄₀ | Cell Wt. | Protein | Sp. Act. | Yield |
|------|------|--------|--------|--------|-------|
| h    | YP Medium | mg/ml | mg/ml | mg/ml | mg/ml |
| 0    | 1.54 | 5.12 | 4.84 | 0.69 | 2.95 |
| 2    | 1.60 | 6.16 | 4.44 | 0.83 | 3.66 |
| 4    | 1.72 | 8.17 | 4.64 | 0.98 | 4.08 |
| 6    | 1.90 | 9.28 | 4.71 | 1.41 | 5.87 |
| 9    | 2.28 | 13.56 | 5.69 | 2.38 | 15.58 |
| 10   | 2.40 | 16.65 | 5.42 | 3.26 | 16.93 |
| 12   | 2.50 | 17.76 | 3.11 | 3.80 | 12.00 |

YP medium = 18 h yeast extract, 2% bacto-peptone.

TABLE II

| Fraction | Specific Activity | Total Volume | Total Protein | Total Activity | Percent Yield | Partition Fold |
|----------|------------------|--------------|---------------|---------------|---------------|---------------|
|          | RLU             | mL           | mg            | RLU           |               |               |
| Clarified Lysozyme | 6.2       | 193          | 2200          | 13640         | 100%          | 1.0           |
| Ammonium Sulfate   | 9.5       | 66           | 1030          | 9785          | 72%           | 1.5           |
| Aff-Gel Blue       | 76.2      | 77           | 72.4          | 5517          | 45%           | 12.3          |
| CM-S2 Cellose      | 102.3    | 85           | 65.5          | 5517          | 49%           | 16.5          |
| YM10 Concenrtrant  | 78.3      | 9            | 53.1          | 4158          | 30%           | 12.6          |
| Dialyzed ODase     | 75.8      | 9            | 53.0          | 4023          | 30%           | 12.2          |

aEnzyme activity was measured at 70°C in Tris-EDTA pH 7.4.
bPercent yield is based on ODase activity recovered.
cpurity of the ODase activity recovered.
dThe ODase activity of the ammonium sulfate fractions loaded was not retained on the Aff-Gel Blue column.
eThe CM-S2 cellulose fractions contain 100 mM Tris-EDTA which inhibits ODase against loss of activity from dilution.

RESULTS

Sequential dialysis. Following purification, ODase was subjected to sequential dialysis to remove residual bound u-UMP. Using 1% PAGE, we determined that 0.65 ml of u-UMP remains bound per ml of ODase dialyzed following dialysis for 1 h against 500 x 10⁷ M sodium phosphate, pH 6.5 containing 5 mM 2-mercaptoethanol and 0.1% glycerol with a buffer change after 1 h. Extensive dialysis for 72 h against a buffer containing 500 x 10⁷ M sodium phosphate and 0.1% glycerol removed 95% of the bound u-UMP. ODase activity was determined by electrophoresis on a 7.5% polyacrylamide gel and stained with Coomassie Blue. The results of the ODase activity assay are shown in Table II. ODase activity was found to be stable at pH 7.5 and 37°C for at least 90% activity remaining after 8 h of storage.

FIG. 1. Preparative of yeast ODase. ODase purified by all Aff-Gel Blue chromatography (Lanes 1 & 2) or by Aff-Gel Blue and CM-S2 cellulose chromatography (Lanes 3, 4 & 5) was run on a 10% SDS-polyacrylamide gel and silver stained. Neither protein included on an ammionium sulfate step or the presence of EDTA in the buffer. The positions of the ODase monomer (a) and the dimer (b) are indicated. Each lane contains 4 μg of ODase stored at -20°C for the indicated times: Lanes 1 & 2 - 1 week; Lanes 3 & 4 - 4 weeks; and Lanes 5 - 5 weeks.

FIG. 2. Removal of residual u-UMP from ODase dialyzed by dialysis. ODase (15 μM dimers) and [3H]-u-UMP (10 μM) in 1.8 ml were mixed overnight at 4°C. Unbound u-UMP was removed by dialysis against 1 liter of 500 x 10⁷ M sodium phosphate, pH 6.5 containing 5 mM 2-mercaptoethanol and 0.1% glycerol with a buffer change after 1 h. Two 5-μl aliquots of the protein solution were removed and counted in 3 ml of Toluene-Solvent Liscum scintillation fluid. The amount of u-UMP measured (42 μM or 0.15 ml u-UMP per ml of enzyme) was taken as the amount bound to the protein in 1.8 ml of solution with no u-UMP in the sample. The data were analyzed by linear regression. The results of the ODase activity were 100% u-UMP and 0.1% u-UMP. ODase activity was found to be 72% with aliquots removed and counted at the time points indicated. Each point is the average of three determinations.

GLYCEROL STABILIZATION. Pure ODase is easily inactivated by freezing (8), therefore, we sought to stabilize the enzyme by including glycerol in the storage buffer. As shown in Fig. 3, the enzyme activity was stabilized at 20°C for more than 2 years by including as little as 10% glycerol. Our purified ODase was routinely stored at 20°C in a buffer containing 500 x 10⁷ M sodium phosphate, 5 mM 2-mercaptoethanol, 20% glycerol, 1 mM EDTA, 1 mM PMSE, 10 μM pyrophosphate A, and 10 μM pyrophosphate B. 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 dialysis solutions with binding of ODase to the Aff-Gel Blue resin or at any other purification step.

FIG. 3. Effect of salts on purification of ODase. ODase purified ODase was stored at 20°C in sodium phosphate buffer, pH 7.5 containing 500 x 10⁷ M sodium phosphate, 5 mM 2-mercaptoethanol, 20% glycerol, 1 mM EDTA, 1 mM PMSE, 10 μM pyrophosphate A, and 10 μM pyrophosphate B. The purified ODase was stored at 20°C in the presence of various concentrations of NaCl, KCl, and Na₂SO₄. The ODase activity was determined by electrophoresis on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Blue. The results of the ODase activity assay are shown in Table II.
Several conflicting K_m values for OMP have been reported for GDCase purified from commercial baker's yeast. Brady and Wendorff (1) reported a K_m for pure GDCase of 1.5 mM OMP measured at 25°C in 5 M sodium phosphate, pH 6.0, and 5 mM 2-mercaptoethanol. Under the same experimental conditions, Linzen et al. (4) reported a K_m of 3.0 mM OMP. At 15°C, in Tris-HCl buffer, pH 7.4, and 1 mM diithiothreitol, Pyle et al. (7) reported two K_m values for yeast GDCase observed as low or high OMP concentrations, a K_m of 0.46 mM when OMP was below 0.3 mM, and a K_m of 2.1 mM when OMP was above 0.3 mM. Brady and Wendorff (1) reported only one K_m value of 0.33 mM under similar experimental conditions with OMP concentrations ranging from 0.3 to 4 mM.

Kinetic parameters for the plankton-derived GDCase were measured in order to compare the values obtained with values reported for the baker's yeast (7). K_m values for yeast GDCase were measured at 25°C in 50 mM sodium phosphate, pH 6.0, and 5 mM 2-mercaptoethanol. We obtained a K_m of 2.6 mM with OMP concentrations ranging from 0.3 to 4 mM. Under the same experimental conditions using MES buffer instead of phosphate, the K_m was 0.7 mM. The V_max for GDCase in both buffers was the same (6000 units/ml), and a double reciprocal plot of the two substrate saturation curves showed competitive inhibition by orthophosphate (Fig. 5). Therefore, the K_m for OMP in the absence of orthophosphate is at 1.1 mM. These data confirm that phosphatase is a competitive inhibitor of GDCase, and the K_i of 3.2 mM for orthophosphate calculated from these data is consistent with an independently determined value of 0.9 mM. The K_m for OMP was also determined in the presence of orthophosphate, and a K_m of 2.9 mM OMP was obtained for GDCase at 0.05 mM orthophosphate. V_max decreased to 20% of the value obtained in the absence of orthophosphate, and the K_m for OMP decreased to 0.6 mM. GDCase is more sensitive to inhibition by orthophosphate than the yeast value due to inhibition by phosphatase. At 15°C in 50 mM Tris-HCl, pH 7.4, a K_m of 2.2 mM was calculated over the OMP range of 0.3 to 40 mM. This value is consistent with the K_m value obtained in high OMP concentrations by Pyle et al. (7), however, we did not observe two K_m values under these conditions.

**FIG. 5.** Competitive inhibition of GDCase by orthophosphate. Each assay contained 50 mM of the indicated buffer at pH 6.5, 5 mM 2-mercaptoethanol, 1.5 x 10^{-5} M GDCase, and 0.25-4.0 mM OMP in a final volume of 0.1 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.