Human Uridine Monophosphate Synthase: Baculovirus Expression, Immunoaffinity Column Purification and Characterization of the Acetylated Amino Terminus†

Byoung-Don Han, Laura R. Livingstone,‡ Daniel A. Pasek, Michael J. Yablonski, and Mary Ellen Jones*

Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260

Received March 7, 1995; Revised Manuscript Received May 30, 1995®

ABSTRACT: Human uridine monophosphate (UMP) synthase, a bifunctional protein containing orotate phsophoribosyltransferase (OPRTase, EC 2.4.2.10) and orotidine 5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23) activities, has been overproduced by construction and use of a recombinant baculovirus containing the cDNA for this protein. Expression of the virus in cabbage looper larvae produces a crude larval homogenate having UMP synthase enriched about 180-fold over human placental homogenates and allows larger quantities of this human protein as well as analog proteins to be prepared for structure/function studies. A vastly improved purification procedure using a monoclonal immunoaffinity column was developed. Human UMP synthase purified from larval extracts yielded a product which comigrates in SDS gel electrophoresis with UMP synthase purified from human placenta; pure proteins prepared from these two tissue sources have the same specific activities. We found that OPRTase requires P_i ions in the assay buffers for optimal OPRTase activity; BSA in the assay vessel increases to a lesser degree both OPRTase and ODCase activities. These changes in the assay are essential to observe a parallel enrichment of the two enzyme activities. The baculovirus system was used to express human UMP synthase because it usually yields a product with posttranslational modifications that reflect those of the organism that provided the cDNA. We report data to show that human UMP synthase derived from either human placenta or larval extracts both have a sequence in which the N-terminal methionine has been removed and the formerly penultimate alanine has been acetylated.

UMP[1] biosynthesis, *de novo*, requires six enzymatic reactions (Jones, 1980). The last two enzymes (OPRTase and ODCase) are present in a bifunctional protein named UMP synthase which occurs in animals (McClard *et al.*, 1980; Livingstone & Jones, 1987) and plants (Walther *et al.*, 1984). This is in contrast to bacteria and fungi, which have two distinct genes and two separable proteins, each of which has one of these two activities.

The available tissue sources for purification of human UMP synthase are erythrocytes or placenta; however, the level of UMP synthase present in these tissues (Livingstone & Jones, 1987) is so low that seven human placentas yield only about 1 mg of pure protein. To have a method that allows many milligrams of pure protein for structure/function studies as well as a method for expressing analog or truncated proteins, a biotechnological method was devised. We, therefore, selected to construct a baculovirus vector carrying the cDNA for human UMP synthase. This system was chosen because unpublished studies in this laboratory by L. R. Livingstone, M. E. Perry, and D. G. Klapper had shown that the N-terminus of UMP synthase was blocked. Baculovirus expression usually produces eukaryotic proteins that are correctly modified with respect to N-terminal processing, phosphorylation, and other types of posttranslational modifications (Luckow, 1991). Experiments reported here show that the N-terminal sequence of pure human UMP synthase is modified *in vivo* and that the changes required from the gene-coded sequence are removal of the N-terminal methionine residue and the subsequent acetylation of the formerly penultimate alanine residue to yield acetyl-AVAR as the acylated N-terminal tetrapeptide. Purified protein produced in cabbage looper larvae expressing the human cDNA was shown to also begin with the N-terminal acetyl-AVAR. The vector overexpressed in intact cabbage looper (*Tricoplusia ni*) larvae produces crude larval extracts having 180-fold[2] more human UMP synthase than placental extracts.

In addition, we have developed a rapid and vastly improved purification procedure which utilizes an affinity column having a monoclonal antibody (prepared to pure human placental UMP synthase) as ligand. We report for the first time that phosphate ion is essential in the assay buffer to observe optimal OPRTase activity and that the use of P_i buffers demonstrates that purification of human UMP syn-

† This research was supported by Grant GM 34539 from the National Institutes of Health and by both a University Council and a Biomedical Research Support Grant from this university.

* To whom correspondence should be addressed.

‡ Present address: Program of Molecular Biology and Biotechnology, CB 7100, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7100.

® Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride, hydrochloride; BCIP, 5-bromo-4-chloro-3-indoyl phosphate toluidine salt; BMP 1-(5′-phospho-β-D-ribofuranosyl)barbturic acid; BSA, bovine serum albumin; CNBr, cyanogen bromide; DIFP, diisopropyl fluorophosphate; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NBT, *p*-nitroblue tetrazoliumchloride; OA, orotic acid; ODCase, orotidine 5′-monophosphate decarboxylase; OMP, orotidylate; OPRTase, orotate phosphoribosyltransferase; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM sodium phosphate−0.15 M NaCl buffer (the pH is noted as required as follows: PBS-8.7 means the pH was adjusted to 8.7); PCR, polymerase chain reaction; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; PRPP, 5-phosphoribosyl 1-pyrophosphate; RT, room temperature (ca. 20−22 °C); SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; UMP, uridine monophosphate.

0006-2960/95/0434-10835$09.00/0

thase yields a product with a parallel enrichment of OPRTase and ODCase activities.

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the indicated sources: collodion dialysis bags (Schleicher & Schuell); human placentas (UNC Memorial Hospital, Chapel Hill, NC); sodium [7-^{14}C]orotate and sodium [7-^{14}C]-OMP (New England Nuclear); PEG 10 000 molecular weight (Fluka); DTT, ultrapure (Boehringer Mannheim); protein A−Sepharose CL-4B, 98−99% pure BSA (product number A 7908), lyophilized soybean trypsin inhibitor, type I-S, iodoacetamide, endoprotease Glu-C (V8 protease), and endoprotease Arg-C (Sigma); cyanogen bromide (Aldrich or Sigma); formic acid, 88% solution, (Mallinckrodt); urea, ultrapure (Schwarz/Mann Biotech); guanidine hydrochloride, ultrapure enzyme grade (Bethesda Research Laboratories); HPLC-grade acetonitrile (Fisher Scientific); HPLC/spectrograde TFA (Pierce); C-8 cartridge HPLC column (Applied Biosystems); and C-18 Nova Pac HPLC column (Millipore). The yeast ODCase used was either the 60−90% ammonium sulfate fraction (for the radioactive assays) or the pure enzyme (for the spectrophotometric assays) produced using the method of Bell and Jones (1991). The N-terminal peptide acetyl-AVAR was synthesized at the Protein Chemistry Laboratory of the University of North Carolina at Chapel Hill−National Institute of Environmental and Health Sciences. All other chemicals were reagent grade or as specified in the appropriate references.

Construction of Recombinant Baculovirus. The entire coding region of UMP synthase plus 209 extra bases of the 3′ noncoding sequence was inserted downstream of the strong polyhedrin promoter of baculovirus by homologous recombination. An *Nco*I site was introduced into the cDNA of human UMP synthase[3] (Suttle *et al.*, 1988) by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987). The oligonucleotide was designed to retain the first amino acid of UMP synthase, a methionine (ACA ATG → ACC ATG). The mutation was confirmed (data not shown) with the dideoxynucleotide sequencing method (Sanger *et al.*, 1977). The 1.8-kb fragment between two *Nco*I sites of the modified UMP synthase cDNA in M13mp19 was subcloned into the *Nco*I site of the pAcC4 baculovirus transfer vector to yield pAcUMPS. The 11.1-kb pAcUMPS was purified by CsCl/ethidium bromide equilibrium centrifugation methods (Ausubel *et al.*, 1989). The purified pAcUMPS was cotransfected with wild-type linear *Autographa californica* nuclear polyhedrosis virus (baculovirus) DNA (Invitrogen) into *Spodoptera frugiperda* cells (*Sf9*) using the procedures from Invitrogen. The recombinant baculovirus from the occlusion-negative plaques were amplified and the following three methods were used to confirm the expression of UMP synthase: (1) PCR amplification using primers derived from the inserted cDNA sequence (ATGGCGGTCGCTCGTGCA for 5′ end and GTCTAGTCCCATGGAGAG for 3′ end) with a DNA thermal cycler (Perkin Elmer Cetus). PCR reactions were performed in a final volume of 100 μL containing 5 mM dNTPs, 5 units of *Taq* polymerase, 1 μM primer pairs, 10 μL of template, and reaction buffer (Boehringer Mannheim). After 50 μL of mineral oil was overlaid the reactions were subjected to 35 cycles of PCR: 45 s at 95 °C to denaturate 30 s at 58 °C to anneal, and 90 s at 72 °C to extend. The amplified products were loaded on an 0.8% agarose gel. (2) Enzyme activities were measured in homogenates of *Sf9* cells carrying the vector. (3) A Western blot was done using antibody prepared against denatured human UMP synthase purified from human placenta (Perry & Jones, 1989). *Trichoplusia ni* (cabbage looper) larvae growth conditions and injection procedures for recombinant baculovirus followed previously published methods (Medin *et al.*, 1990). Other procedures followed a manual of methods for baculovirus vectors and cell culture by Summer and Smith (1987).

Preparation of the Monoclonal Immunoaffinity Column. Monoclonal antibody 26 (IgG_1 subclass) was made by the immunization of mice to pure native human placental UMP synthase, and cells producing antibody 26 were grown in culture as previously described (Livingstone, 1984). Culture medium containing antibody 26 was collected, centrifuged to remove cell debris, and stored frozen at −20 °C. Purification of antibody 26 was by a modification of the procedure of Bigbee *et al.* (1983). Antibody 26 was attached to Affi-Prep 10 resin using a concentration of 12 mg of antibody/mL of resin following the protocol of the manufacturer (Bio-Rad). The resin was stored in PBS-7.2 at 4 °C plus 0.05% sodium azide and was used repeatedly for human UMP synthase purification with no apparent loss of affinity for human UMP synthase from placental extracts. However, some loss of affinity was observed after repeated use purifying human protein from larval extracts.

Composition of Buffers for Purification of Human UMP Synthase. The following buffers were used in the immunoaffinity purification of UMP synthase from placenta: (1) phosphate buffer, 20 mM potassium phosphate buffer, pH 7.3 at 0 °C; (2) ethylene glycol wash buffer, 50% ethylene glycol−20 mM potassium phosphate buffer, pH 7.3 at 0 °C; (3) NaCl wash buffer, 1 M NaCl−20 mM potassium phosphate buffer, pH 7.3 at 0 °C; (4) $MgCl_2$ wash buffer, 0.2 M $MgCl_2$−20 mM MOPS-KOH, pH 7.2 at 0 °C, plus 0.02% PEG 10 000; (5) elution buffer, 3 M $MgCl_2$−20 mM MOPS-KOH, pH 7.0 at 0 °C, plus 0.02% PEG 10 000; (6) recycling buffer, 3 M $MgCl_2$−20 mM MOPS-KOH, pH 3.5 at 0 °C, plus 0.02% PEG 10 000; (7) neutralization buffer, 1 M MOPS-KOH, pH 8.0 at 0 °C; (8) dialysis buffer 1, 20 mM MOPS-KOH, pH 7.0 at 0 °C, plus 0.02% PEG 10 000; and (9) dialysis buffer 2, 40% glycerol−20 mM potassium phosphate, pH 7.3 at 0 °C, plus 0.02% PEG 10 000. All of the above buffers contained 2 mM DTT.

For purification of human UMP synthase from larvae, buffers 1−3 and 8−9 were changed to pH 6.8 at 0 °C. Buffer 5 was changed to pH 6.8 at 0 °C with the addition of 1 mM EDTA, 5 μg/mL pepstatin, 1 mM AEBSF (sold as Pefabloc SC by Boehringer Mannheim), and 10 mM DIFP. Buffer 4 was changed to pH 7.0 at 0 °C; buffers 6 and 7 remained at pH 3.5 and 8.0, respectively.

PEG was omitted from all buffers for purifications of human UMP synthase used for the amino-terminal characterization. Following purification on the immunoaffinity

[2] We have compared OPRTase purification values because proteolysis of UMP synthase usually destroys this enzyme domain rapidly to produce the relatively stable ODCase domain (MW ca. 28 500). Therefore, during purification of UMP synthase, ODCase yields have previously been higher than OPRTase yields (Floyd & Jones, 1985); this situation was also exaggerated by the use of Tris buffers, which reduced the "apparent" OPRTase activity. The latter situation has been improved, as described in this paper by the addition of P_i ion to assay buffers.

[3] cDNA of human UMP synthase was kindly provided by Dr. D. Parker Suttle, Department of Pharmacology, University of Tennessee, 874 Union Ave., Memphis, TN 38163.

column, the UMP synthase preparations were dialyzed in a collodion bag against distilled water. The protein sample was then dried in preparation for CNBr cleavage.

Immunoaffinity Purification of Human UMP Synthase. Placentas were processed and stored as resuspended ammonium sulfate pellets (in 20 mM potassium phosphate, pH 7.3 at 0 °C, plus 2 mM DTT, 1 mM EDTA, 10 μg/mL pepstatin, and 40 μg/mL leupeptin) as previously described (Livingstone & Jones, 1987). Resuspended ammonium sulfate pellets representing about 16 000 nmol/min ODCase activity (usually about 300 mL) were thawed, centrifuged at 27000*g* for 15 min to remove precipitate, and loaded (flow rate of 394 mL/h) onto an 8-mL column (2.5-cm diameter × 1.63-cm height) of monoclonal antibody 26−Affi-Prep 10 resin equilibrated in phosphate buffer via a GF/D glass filter (Whatman) to prevent column clogging. The column was washed with 200 mL of phosphate buffer (394 mL/h), 240 mL of ethylene glycol wash buffer (394 mL/h), 200 mL of NaCl wash buffer (394 mL/h), 100 mL of phosphate buffer (394 mL/h), 120 mL of $MgCl_2$ wash buffer (394 mL/h), 16 mL of $MgCl_2$ wash buffer (39.4 mL/h), 20 mL of elution buffer (39.4 mL/h), 27 mL of recycling buffer (394 mL/h), and 100 mL of phosphate buffer (394 mL/h). Fractions collected containing the elution or recycling buffers were immediately neutralized to about pH 7 addition of neutralization buffer. Fractions containing UMP synthase were detected by using absorbance at 280 nm and by using the ODCase spectrophotometric assay (Silva & Hatfield, 1978) at RT with the addition of 2 mM DTT and 0.1 mM EDTA. These neutralized fractions were pooled and concentrated with an 8-mL capacity 25 000 molecular weight cutoff collodion bag and dialyzed against dialysis buffer 1 (2 × 550 mL for 45 min) and dialysis buffer 2 (1 × 550 mL for 90 min). The concentrated enzyme was removed, the collodion bag was rinsed with dialysis buffer 2, and the rinses were added to the concentrated enzyme until the total volume was 2 mL. The pure UMP synthase is optimally stored in small aliquots frozen at −70 °C.

Larvae were collected and frozen at −70 °C 5 days after injection with recombinant virus. Frozen larvae were homogenized with a rotor-stator UT-dispersing tool T25 (IKA-Works) using a buffer (50 mM potassium phosphate buffer, pH 7.0 at 25 °C, containing 2 mM DTT, 2 mM EDTA, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 mM DIFP, 1 mM PMSF, and 100 μg/mL soybean trypsin inhibitor). Note that DIFP is a highly toxic cholinesterase inhibitor that may be fatal if inhaled, swallowed, or absorbed through skin and thus should be handled with appropriate care. The antidote is atropine sulfate and 2-PAM (2-pyridinealdoxime methiodide). We use a strong base to hydrolyze DIFP in solutions of preparation materials that are to be discarded. Four milliliters of the homogenization buffer were used/g of larvae and this was pooled with 1 mL of buffer/g of larvae used to rinse the homogenizer. After centrifugation of the homogenate for 20 min at 30000*g* at 4 °C, the supernatant was fractionated with ammonium sulfate and the protein precipitating between 35% and 55% ammonium sulfate saturation was collected. The precipitate was resuspended in a minimal volume of homogenization buffer and stored at −70 °C. About 1.5 mL of this suspension was applied to an immunoaffinity column as described for the purification of human UMP synthase from placenta. The purification followed procedures for placental extracts except for the changes in the buffers (see above) and a flow rate of 163 mL/h for the loading of the resuspended ammonium sulfate pellets. Protease inhibitors at concentrations of 5 μg/mL pepstatin, 1 mM AEBSF, and 10 mM DIFP were added to the final concentrated product.

Enzyme Assays of Human UMP Synthase. ODCase activity was determined at 37 °C by measuring the release of $^{14}CO_2$ from [^{14}C]OMP at previously described (Jones *et al.*, 1978). OPRTase activity was determined at 37 °C by measuring the release of $^{14}CO_2$ from [^{14}C]OA after sequential conversion to OMP and UMP. The double-stopped OPRTase assay was routinely used (Alvarado *et al.*, 1988) where the conversion of OA and PRPP to OMP was halted by the addition of EDTA (to chelate Mg^{2+}) plus yeast ODCase to allow conversion of accumulated OMP to UMP before addition of acid to release $^{14}CO_2$. The specific activities of the [^{14}C]OMP or [^{14}C]orotate in the reaction mixtures were determined by adding large excesses of UMP synthase to allow total conversion of substrate. Assay mixtures (pH 7.4 at 37 °C) contained 20 mM Tris-HCl and 0.1 mM EDTA with either 5 mM $MgCl_2$, 0.3 mM PRPP, 100 μM [7-^{14}C]-orotic acid (1−3 Ci/mol), and 2 mM DTT (OPRTase assays) or 40 μM [7-^{14}C]OMP (1−3 Ci/mol) (ODCase assays). The assay conditions for the pure enzyme were modified and are described in Table 2. The assay mixture volume, assay time, and quantity of enzyme were adjusted so that OPRTase and the ODCase assays typically converted less than 15% and 50% of the substrate, respectively.

Human UMP synthase activities from larvae were measured using similar procedures as for UMP synthase from placenta. The radioactive OPRTase assay included the 60−90% ammonium sulfate fraction of yeast ODCase (Bell & Jones, 1991), which has no OPRTase activity, in the assay mixture at the start of the assay.

Spectrophotometric assays, modified from the protocols of Silva and Hatfield (1978), were used to determine ODCase and OPRTase activities in Tables 1 and 2. The ODCase assay mixture consisted of 10−15 μg/mL pure UMP synthase, 15 mM MOPS (pH 7.0 at RT), 5 mM potassium phosphate (pH 7.0 at RT), 2 mM DTT, 0.1 mM EDTA, and 200 μM OMP. The OPRTase assay mixture consisted of 10−15 μg/mL pure UMP synthase, 15 mM MOPS (pH 7.0 at RT), 5 mM potassium phosphate (pH 7.0 at RT), 2 mM DTT, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.3 mM PRPP, 100 μM OA, and pure yeast ODCase (Bell & Jones, 1991) at about 10 times the activity (nmol/min) of the UMP synthase. We found that maximum ODCase activity was observed when we used the concentration of UMP synthase stated above; the maximum OPRTase activity was observed when the amount of yeast ODCase stated above was added (data not shown). The absorbance of the assay mixture prior to the addition of OA or OMP was used as the reference for the assay. OA or OMP was then added and mixed to start the reaction. After 10 s the reaction was followed at RT using a Spectronic 3000 array spectrophotometer (Milton Roy). ODCase assays were measured at 285 nm and OPRTase assays at 295 nm. The ODCase reaction was linear until the remaining OMP is about 10 μM (data not shown). Reaction rates were determined from a time interval within the linear range. Initial rates for the reversible OPRTase reaction can only be observed during early time intervals (data not shown); therefore, the initial 25 s (10−35 s after the addition of substrate) were used.

The data for Figure 3 were collected spectrophotometrically as described above except that the OPRTase assay

mixture did not contain any yeast ODCase or EDTA. The P_i concentration was varied between 0.25 and 200 mM, and the assays were done at 37 °C (controlled with a thermoelectric cuvette holder).

Protein Determination. Protein concentrations were determined by the method of Bradford (1976) using a bovine serum albumin standard calibrated using the absorption value of 3.03 for a 1% BSA solution (1-cm cell) at 278 nm (Kirschenbaum, 1973).

SDS Gel Electrophoresis and Western Blotting. The procedure of Laemmli (1970) was used for all SDS gel electrophoresis using 12% acrylamide, 0.75-mm-thick slab gels that were silver-stained as previously described (Livingstone & Jones, 1987). Low molecular weight protein standards (Bio-Rad or Sigma) were used for molecular weight calibrations. Proteins from the gel were transferred to nitrocellulose paper (Schleicher & Schuell) by a semidry electrophoretic transfer unit (BioTrans Model B, Gelman Sciences). For immunodetection of UMP synthase, a polyclonal rabbit anti-human placenta UMP synthase serum produced against denatured human UMP synthase (Perry & Jones, 1989) was applied as the first antibody. This antibody was then detected with goat anti-rabbit IgG conjugated with alkaline phosphatase and color was developed with NBT and BCIP as described in the manufacturer's protocol (Bio-Rad).

Scanning of Stained Gels. Densitometric scans of stained gels and integrations of peak areas were done using a Zeineh soft laser scanning densitometer Model SLR-2D/1D (Biomed Instruments) to determine relative amounts of protein in stained bands. The linear band density ranges were calculated by determining the density of the bands at several protein concentrations (as in Figure 2).

Cleavage of UMP Synthase with CNBr and Endoprotease Glu-C (V8 Protease). The dried protein was resuspended in 70% formic acid and was evaporated to dryness. Cleavage at methionine residues by CNBr followed a modification of the conditions reviewed by Gross (1967). The sample was dissolved to a protein concentration of 8 mg/mL in 70% formic acid containing a 110-fold excess of CNBr (over methionine) and 3 mM DTT. Cleavage was for 24 h at RT in the dark. The solution was evaporated to dryness three times from 70% formic acid to remove volatile reaction products. The sample was dissolved in 70% formic acid and transferred to a glass vial. To remove formic acid, the sample was dried three times from distilled water. The sample was cleaved by V8 protease using the procedure of Drapeau (1977). Dried peptides were dissolved in 1 M ammonium acetate, pH 4.0, containing 6 M guanidine hydrochloride and 3 mM DTT. This solution was diluted to yield 1 M guanidine by adding ammonium acetate/DTT buffer. V8 protease, freshly dissolved to 1 mg/mL in distilled water, was added at a ratio of 1/60 protease/peptides by mass. The mixture was digested at RT for 24 h with agitation. The resulting peptides were dried three times from distilled water to remove ammonium acetate. Aliquots of the peptides taken after cleavage were separated on a high-Tris SDS−polyacrylamide gel (Fling & Gregerson, 1986) with a gradient of 15−25% polyacrylamide in order to determine the extent of protein cleavage.

Alkylation of Cysteines. After fragmentation into peptides, cysteines were reduced with DTT (Konigsberg, 1972) and alkylated with iodoacetamide (Gurd, 1972). The sample was dissolved to a protein concentration of 50 mg/mL in 0.5 M HEPES, pH 8.2, containing 8 M urea and incubated with a 50-fold excess of DTT over cysteine for 3.5 h at RT. A 55-fold excess of iodoacetamide was added and incubated for a further 1.5 h at RT in the dark. The reaction was stopped by adding a 2-fold excess of β-mercaptoethanol and the sample was then stored at −20 °C. All solvents and reaction products could be removed by subsequent reverse-phase HPLC.

HPLC Isolation of an N-Terminal Peptide Terminating at Met 93. Following reduction and alkylation, the peptides were diluted to 1−5 mg/mL in 6 M guanidine hydrochloride and 100 μL was injected onto the HPLC column. Peptides were separated on a Brownlee Spheri-5 RP-8 (C-8) column with dimensions of 30 × 4.6 mm and a particle size of 5 μm. The elution solvents were water and acetonitrile, both containing 0.1% TFA (v/v), and peptides were eluted by a gradient of 0−60% acetonitrile at a flow rate of 0.5 mL/min. Elution was monitored at 214 nm in a detector cell volume of 8 μL. The large peak eluting at about 57% acetonitrile was collected and a small aliquot was taken directly from the elution solvent, dried three times from distilled water, and stored at −20 °C for analysis by amino acid composition and SDS electrophoresis. The remaining peptide was dried three times from water and resuspended in 6 M guanidine hydrochloride for HPLC. This peptide was further purified by chromatography on a Waters C-18 Nova Pak column of particle size 4 μm, pore size 60 Å, and dimensions of 2 × 150 mm using a gradient of 0−80% acetonitrile. The peak eluting at about 67% acetonitrile was concentrated in a glass vial and treated as above in preparation for cleavage by endoprotease Arg-C.

Production of a Short N-Terminal Peptide Terminating at Arg 5. Fragmentation by endoprotease Arg-C followed the procedure of Levy *et al.* (1970). The purified long N-terminal peptide was dissolved in 1 M ammonium bicarbonate, pH 8.0, containing 8 M urea at a protein concentration of 8 mg/mL. The urea was then diluted to 2 M by the addition of ammonium bicarbonate buffer. Endoprotease Arg-C, freshly dissolved in 1 M ammonium bicarbonate buffer at a concentration of 1 mg/mL, was added to the peptides at a ratio of 1/500 by mass of protease/peptide substrate. The digestion was 24 h at 37 °C with gentle agitation. To check the digestion, an aliquot was evaporated to dryness and dissolved in 6 M guanidine hydrochloride for analysis by HPLC.

HPLC Purification of the Short N-Terminal Peptide. The peptides resulting from digestion by Arg-C were dried 3 times from water and resuspended in 6 M guanidine hydrochloride to a protein concentration of 1 mg/mL. The sample (100 μL) was then injected onto the C-18 HPLC column. The peptides were separated by a gradient of 0−80% acetonitrile at a flow rate of 0.2 mL/min. All clearly resolved peaks were dried three times from water and stored at −20 °C. The first peak to elute (at about 16% acetonitrile) was expected to be the N-terminal peptide and was analyzed by amino acid analysis (data not shown) and mass spectrometry.

Mass Spectrometry Analysis. The purified N-terminal peptide was redissolved in distilled water, divided into aliquots, dried under vacuum, and stored at −20 °C. The peptide was dissolved in thioglycerol immediately before analysis by mass spectrometry. The analysis consisted of mass determination on 200−2000 pmol by positive-ion fast-atom bombardment utilizing Xe at 6 kV, an accelerating voltage of 10 keV and a mass resolution of 1000 m/m. The

structure of the peptide was verified by collision-induced dissociation on 1000−2000 pmol of the precursor ion (*m/z* at 458.5) followed by a *B/E*-linked scan of the fragment ions produced. The mass spectrometry analysis was performed at the North Carolina State University Mass Spectrometry Laboratory for Biotechnology Research on a JEOL HX110HF double-focusing mass spectrometer equipped with a high-field homogeneous magnet and DEC LSI 11/73 computer.

RESULTS

Expression of Human UMP Synthase Using the Baculovirus Expression System. We elected to use a recombinant baculovirus (Summer & Smith, 1987) carrying the cDNA for human UMP synthase for overexpression in intact cabbage looper (*T. ni*) larvae (Medin *et al.*, 1990). On the basis of ODCase activity (data not shown), the expression of human UMP synthase in *Sf9* cells reached a maximum on the third day postinfection, while in larvae injected with recombinant baculovirus it continued to increase until harvest on day 5, the day before many larvae die. Human UMP synthase expressed in *Sf9* cells or cabbage looper larvae showed the same molecular weight as the protein from human placenta (51 000−53 000) and comigrate on a 12% SDS-polyacrylamide gel (data not shown). The endogeneous insect ODCase activity in noninfected *Sf9* cells, *Sf9* cells infected with wild-type baculovirus, or in cabbage looper larval extracts does not cross-react either with the monoclonal antibody 26 to human UMP synthase or in Western blots to a polyclonal antibody toward denatured pure human UMP synthase (data not shown).

Immunoaffinity Purification of Human UMP Synthase. A crude placental extract from nearly 3000 g of human placenta, prepared as described by Livingstone and Jones (1987) and containing pepstatin and leupeptin, was applied to the monoclonal antibody column and gave the elution profile for UMP synthase bound shown in Figure 1; a representative purification is summarized in Table 1. Human UMP synthase was also purified with the monoclonal affinity column (Table 1) from less than 4 g of cabbage looper larvae infected wth recombinant baculovirus carrying the cDNA for human UMP synthase. To reduce proteolysis of UMP synthase in larval extracts, the extraction buffer pH was adjusted to 7.0. Protease inhibitors were added to the homogenization and elution 5 buffers as well as to the final concentrated product to preserve intact UMP synthase. Two larval purification protocols are reported (Table 1 and Figure 2). For Figure 2A the protocol used the immunoaffinity column buffers given for the placental purification except that the protease inhibitors specifically selected for the larval extract proteases were used in purifying UMP synthase from larval homogenates. This same protocol was used for Figure 2B except that the more acidic buffers given in Experimental Procedures for the larval purification were used.

Pure human UMP synthase is enriched 7000-fold over the placental extracts or 35-fold over the larval homogenate using OPRTase activity as measured in Tris buffer.[3] UMP synthase was greater than 96% pure from placental extracts, and with the more acidic buffers, the UMP synthase from cabbage looper larvae had a purity of about 90% as determined by densitometric scanning of SDS gels. A typical preparation from larvae (Figure 2B) had several proteins with a molecular weight higher than 51 000−53 000 that make

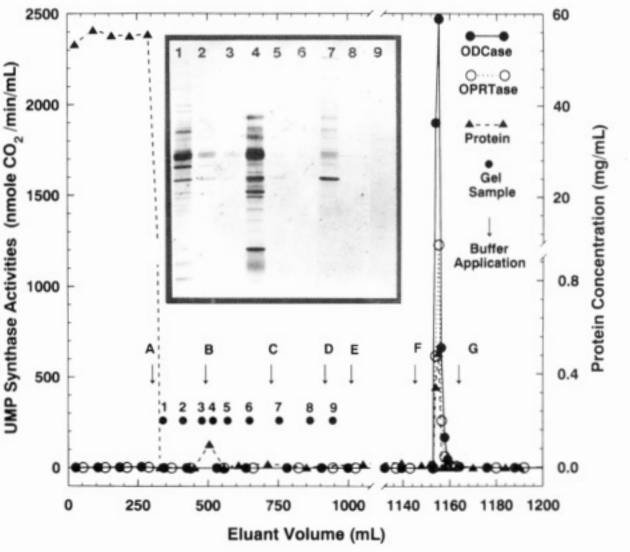

FIGURE 1: Immunoaffinity purification of human UMP synthase from placenta. Human placental UMP synthase was purified using a monoclonal 26 immunoaffinity column as described in the Experimental Procedures and Results. Eluted fractions were measured for ODCase, OPRTase, and protein levels. The arrows represent the start of the application of the following buffers: phosphate buffer (A and D), ethylene glycol wash buffer (B), NaCl wash buffer (C), $MgCl_2$ wash buffer (E), elution buffer (F), and recycling buffer (G). The numbered dots indicate positions at which constant volume aliquots were taken and run on an SDS gel shown in the lane of matching number (see inset, which shows the proteins removed by each buffer prior to elution of pure UMP synthase). Gel electrophoresis and protein visualization by silver stain were as described in Experimental Procedures.

up about 4% of the total protein, and about 6% of the proteins had a molecular weight lower than the UMP synthase. With the addition of protease inhibitors as above but with the column and dialysis buffers at the higher pH values used for the placental purification, the UMP synthase purified from cabbage looper larvae had a purity of only 86% (Figure 2A). The bands in Figure 2A with molecular weights higher than UMP synthase are still 4% of the total proteins, but the lower molecular weight proteins now make up about 10% of the total proteins and may represent mainly proteolyzed products from UMP synthase. The contaminant whose molecular weight is slightly less than 29 000 was shown by Western blot to cross-react with the antibody to denatured human UMP synthase (data now shown) and is the same molecular size that Floyd and Jones (1985) found for the ODCase domain produced by either elastase or trypsin digestion of pure mouse Ehrlich ascites cell UMP synthase.

Necessity of Phosphate Ion for Optimal OPRTase Activity. Human UMP synthases purified from either placental or larval extracts have similar OPRTase and ODCase activities when assayed under the various conditions used in Tables 1 and 2. However, the actual activities observed for both UMP synthases depended, in a predictable way, on the nature of the buffer ion present and/or the presence or absence of BSA. The OPRTase activity of UMP synthase is very dependent on the presence of phosphate ion (Table 2 and Figure 3), while both OPRTase and ODCase benefit from the presence of BSA (Table 2). The various assay conditions used in Table 2 were all identical except for the buffer used and the presence or absence of BSA.

Isolation of N-Terminal Peptides from Human UMP Synthase. In order to expedite the purification of a short N-terminal fragment of UMP synthase, a cleavage strategy

Table 1: Purification of Human UMP Synthase

fraction	volume (mL)	protein (mg)	assay method[a]	orotidine 5′-monophosphate decarboxylase: total activity[b]	orotidine 5′-monophosphate decarboxylase: specific activity[c]	orotidine 5′-monophosphate decarboxylase: yield (%)	orotate phosphoribosyltransferase: total activity[b]	orotate phosphoribosyltransferase: specific activity[c]	orotate phosphoribosyltransferase: yield (%)	ODCase/ OPRTase
Placental Purification										
supernatant of crude homogenate[d]	4060	63990	R	18060	0.28	100	10010	0.16	100	1.8
resuspended ammonium sulfate pellet[d]	330	16220	R	17590	1.08	97	12650	0.78	126	1.4
UMP synthase concentrated immunoaffinity column peak	2.1	1.13	R−EDTA	7070	6260	39	1250	1110	12	5.6
Larval Purification										
supernatant of crude homogenate[e]	16.5	118	R		[109]	100		[29.0]	100	
resuspended ammonium sulfate pellet loaded on column[f]	1.5	57.5	R	15200	264	118	3050	53	89	5.0
UMP synthase concentrated immunoaffinity column peak[g]	2.2	0.54	R	5380	9970 ± 318	42	578	1070 ± 63	17	9.3
			S	3400	6290 ± 88		1470	2720 ± 20		2.3

[a] Assay methods were as described in Experimental Procedures using the following: R = radioactive assays (37 °C) using Tris buffer, 20 mM, pH 7.4, with 0.1 mM EDTA (R) or without EDTA (R − EDTA); S = spectrophotometric assays (ca. 20−22 °C) using a 15 mM MOPS plus 5 mM phosphate buffer, pH 7.0. Both assay methods (R and S) used for the larval purification data include yeast ODCase added to the assay mixture at the beginning of the OPRTase assays. Average errors are included for human UMP synthase immunoaffinity purified from larvae for comparisons between assay methods R (6 values each) and S (4 values each). [b] Nanomoles of CO_2 per minute. [c] Nanomoles of CO_2 per minute per milligram of protein. [d] Values are sums of seven placenta preparations totaling 2970 g. [e] Activity values for this purification step are not available. Yields were, therefore, based on the average of three later batches of larvae using the identical ammonium sulfate purification procedure. The volume (milliliters) and protein (milligrams) are adjusted relative to the size of the resuspended pellet aliquots used in the column purification (see footnote *f*). The crude homogenate supernatant from 40.0 g of larvae was 204 mL and had 1460 mg of protein. [f] The resuspended ammonium sulfate pellet of the crude homogenate (204 mL) yielded 18.5 mL of protein solution of which only 1.5 mL was used for each of two column purification. [g] Values are an average of duplicate purifications.

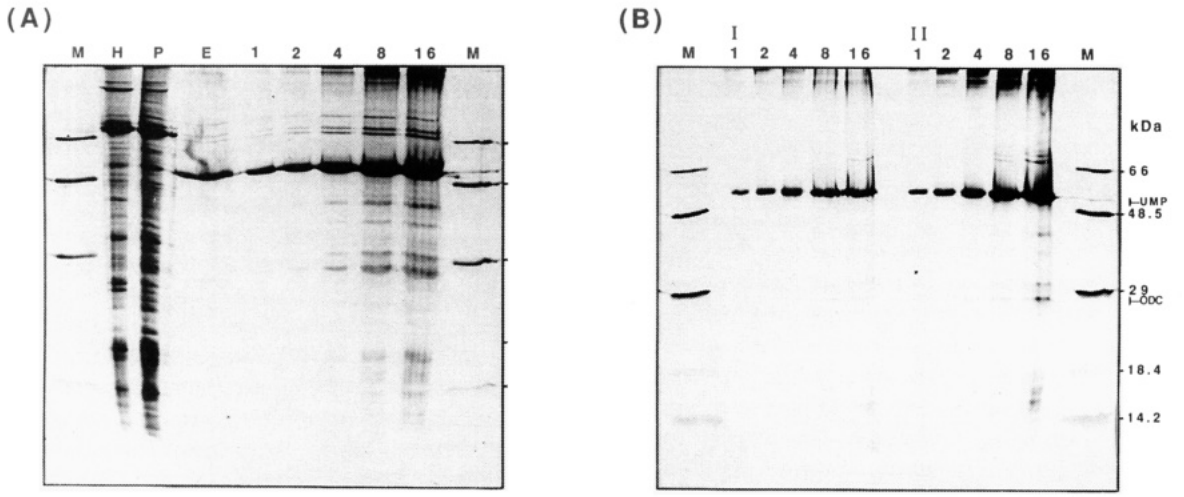

FIGURE 2: SDS gels of human UMP synthase purified from recombinant baculovirus infected larvae. Protein applied was as follows: (A) Purification using column and dialysis buffers at the pH values used for the placenta purification plus the protease inhibitors used for larval extracts in Experimental Procedures. Gel includes samples from various stages of the purification. (B) Protein from duplicate purifications (I and II) that followed the larval purification procedures as given in Experimental Procedures. M, Sigma low molecular weight markers (values in kilodaltons are indicated). H, 7.2 μg of crude homogenate supernatant. P, 19 μg of resuspended ammonium sulfate pellet. E, 2.3 μg of UMP synthase eluted from antibody column; this fraction still contains the 3 M $MgCl_2$ used to elute UMP synthase from the column. 1, 2, 4, 8, and 16 indicate the multiple of the amount of pure UMP synthase loaded. For gel A, 1 = 0.96 μg; for gel B, 1 = 0.59 μg for both preparations I and II. Arrows show UMP synthase (UMP) and putative ODCase domain (ODC).

was devised to first produce a long N-terminal peptide. Because of the cDNA sequence (Suttle *et al.*, 1988), cyanogen bromide was used for the first cleavage at methionine residues, followed by endoprotease Glu-C (V8 protease) cleavage at glutamic acid residues. Assuming that all target residues were cleaved, the N-terminal peptide would terminate at Met 93 and be the longest and most hydrophobic peptide in the mixture.

The long N-terminal peptide isolated by reverse-phase HPLC eluted as a single peak that was well separated from other peaks (data not shown). An amino acid analysis of the peptide was consistent with the amino acid content predicted by the sequence (Klapper, data not shown). Upon electrophoresis on a gradient gel, the peptide gave a single band that had the same mobility as a major band that persisted in the mixture of CNBr peptides and also in the mixture of peptides produced by V8 protease digestion (data not shown). This peptide was then subjected to rechromatography on a C-18 HPLC column and the elution profile showed a major peak which elutes at a high acetonitrile concentration (data not shown).

Further fragmentation of the purified N-terminal peptide at arginine residues produced a short polar N-terminal peptide by cleavage at Arg 5 that was easily isolated by reverse-phase HPLC. The elution profile of the C-18 HPLC column is consistent with the pattern of peptides that would be

Table 2: Comparison of the Specific Activities of OPRTase and ODCase of Pure Human UMP Synthase Concentrated Immunoaffinity Column Peak

		orotidine 5′-monophosphate decarboxylase		orotate phosphoribosyltransferase			
	assay mix[a]	specific activity,[b] placental purification[c]	specific activity,[b] larval purification[c]	specific activity,[b] placental purification[c]	specific activity,[b] larval purification[c]	ODCase/ OPRTase, placental purification[c]	ODCase/ OPRTase, larval purification[c]
radioactive assays (at 37 °C)	T	6640	6590	1260	1290	5.3	5.1
	T/BSA	8290	9820	1640	1670	5.0	5.9
	P	5260	6350	3000	2720	1.8	2.3
	P/BSA	8190	8600	4690	3990	1.8	1.8
spectrophotometric assays (at RT, ca. 20−22 °C)		5760	5560	2840	2440	2.0	2.3

[a] Compositions of assay mixtures were as described in Experimental Procedures using the following buffers without EDTA: T = Tris buffer, 20 mM, pH 7.4; T/BSA = Tris buffer plus 0.1 mg/mL BSA; P = phosphate buffer, 20 mM, pH 7.4; P/BSA = phosphate buffer plus 0.1 mg/mL BSA. BSA was tested for OPRTase and ODCase activities and had neither. Spectrophotometric assay mixtures used 15 mM MOPS and 5 mM phosphate, pH 7.0, as described in Experimental Procedures. [b] Nanomoles of CO_2 per minute per milligram of protein. [c] Placental and larval purifications both used column and dialysis buffers at the pH values described in Experimental Procedures for the placenta purification.

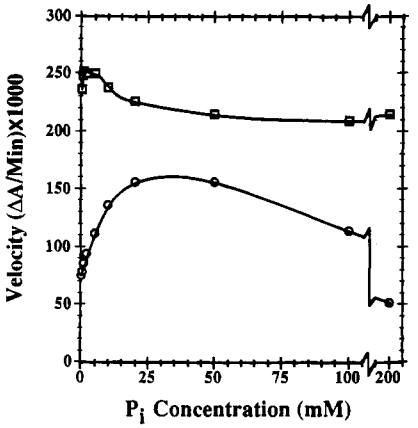

FIGURE 3: Phosphate concentration curves for pure UMP synthase activities. Data were collected at 37 °C using the spectrophotometric assays for ODCase and OPRTase activities as described in Experimental Procedures and had 9.03 μg of human UMP synthase purified from larvae in 0.75 mL of an assay mixture with 15 mM MOPS, pH 7.0, at RT. Reaction rates over the initial 1 min for ODCase (squares) and OPRTase (circles) are plotted vs P_i concentration for 0.25, 0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100, and 200 mM. Conditions for all data points were identical except for P_i concentration.

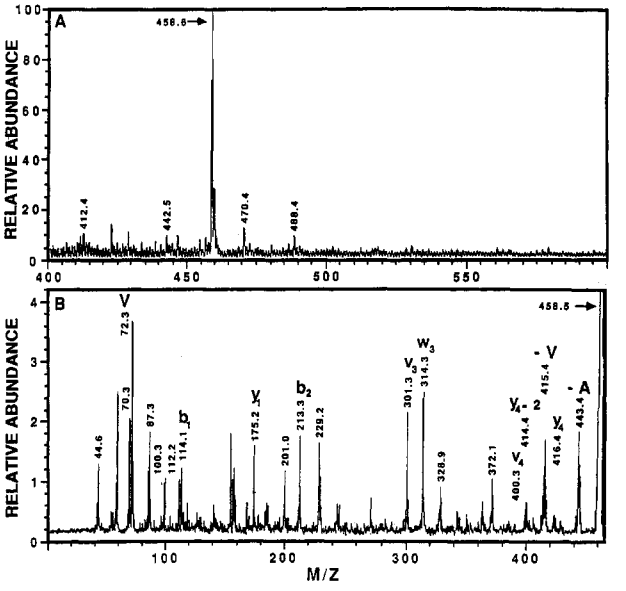

FIGURE 4: Mass spectroscopic analysis of the short N-terminal peptide (acetyl-AVAR) of human UMP synthase. The short N-terminal peptide which had been isolated by HPLC was analyzed by mass spectroscopy as described in Experimental Procedures. Panel A shows the result of a mass determination by positive-ion fast-atom bombardment. The arrow shows the position of the protonated peptide ion with a mass of 458.6. Panel B shows the spectrum resulting from fragmentation of the peptide by collision-induced dissociation (CID). Peak labels for the CID spectrum designate fragmentation of the peptide as follows [see Biemann (1990) for a detailed explanation of the notation]: −A (443.4), loss of an alanine side chain; −V (415.4), loss of a valine side chain; V (72.3), the valine immonium ion; b_1 (114.1) and b_2 (213.3), N-terminal fragments; y_1 (175.2), $y_4 - 2$ (414.4) and y_4 (416.4), C-terminal fragments; v_3 (301.3), C-terminal fragment with loss of a valine side chain; v_4 (400.3), C-terminal fragment with a loss of an alanine side chain; w_3 (314.3), C-terminal fragment with a loss of part of the valine side chain at the β,γ-bond. The spectra shown in both panels are for the UMP synthase which was purified from human placenta tissue. Mass determinations and CID spectra for both the chemically synthesized peptide (acetyl-AVAR) and the N-terminal peptide from human UMP synthase expressed in the baculovirus system were essentially identical (not shown).

expected from a partially incomplete digestion at arginine residues (data not shown).

Since the sequence of the N-terminus is known from the cDNA and since the N-terminal sequence MAVAR might become acetyl-AVAR if the protein is acetylated (Tsunasawa *et al.*, 1985), a peptide standard with the sequence acetyl-AVAR was synthesized by chemical methods. When the standard peptide was mixed with the peptides resulting from digestion at arginine, the standard peptide coeluted at 14.5 min with the smallest peptide from the UMP synthase (data not shown).

Mass Spectroscopy of the N-Terminal Peptide. A mass determination of the purified N-terminal peptide from placental UMP synthase by fast-atom bombardment mass spectroscopy gave 458.501 as the major peak (Figure 4), which agrees with the calculated mass of the peptide acetyl-AVAR. Also, collision-induced dissociation (CID) of this parent ion gave a pattern of fragments that verifies the proposed structure (Figure 4). The fragmentation pattern was interpreted on the basis of previous CID spectra as reviewed by Biemann (1990). Major peaks in the spectrum which result from fragmentation of the parent ion were interpreted as listed in Figure 4. The mass spectrometry and CID spectrum analyses for the chemically synthesized acetyl-AVAR and the N-terminal peptide from pure human UMP

synthase from placental or insect larval extracts are all essentially identical (data not shown).

DISCUSSION

Purifications of UMP synthase to homogeneity from extracts of mouse Ehrlich ascites carcinoma (McClard *et al.*, 1980) and human placenta (Livingstone & Jones, 1987) using chemical affinity columns have been published. In both cases the apparent OPRTase recoveries were very low (about 2%) while ODCase recoveries were between 10% and 30%. Purification of human UMP synthase was lengthy and required 1.5 kg of tissue, which was inconvenient to process and store and yielded only 0.15 mg of pure protein. Human placentas are in demand and are difficult to obtain in the quantities required to purify large amounts of UMP synthase.

In an attempt to purify undenatured human UMP synthase to (1) obtain a better recovery of enzyme activities, (2) be relieved of collecting human tissues, (3) have a method for obtaining analog or truncated proteins, and (4) have large amounts of pure protein for structure/function studies, we investigated the use of a baculovirus vector to produce an insect extract enriched in human UMP synthase. A new monoclonal antibody immunoaffinity column allows us to purify human UMP synthase from these extracts. This purification, and the development of new enzyme assay conditions, has lead to a pure protein from placental or larval extracts with a 50% recovery of both enzyme activities.

A very pure protein was obtained from homogenates of human placenta and a similar purity was obtained for human UMP synthase isolated from cabbage looper larvae infected with a baculoviral vector carrying the cDNA for this protein (Table 1 and Figure 2). The specific activity of the larval homogenate is about 180-fold higher[3] than that of human placental homogenates (Table 1). Monoclonal antibody 26 bound as ligand to the column matrix does not recognize insect UMP synthase, so only human UMP synthase is retained on this column.

The exact amount of the two enzyme activities of pure UMP synthase and their ratio (Table 2) varies with the assay buffer (Tris or P_i); P_i ion is essential for optimal OPRTase activity (see Figure 3). The presence of BSA enhances both OPRTase and ODCase activities. The apparent OPRTase activity as assayed in Tris buffer gave a yield of about 15% (see Table 1), which becomes about a 50% yield when the activity is measured in a buffer containing phosphate ions plus BSA (Table 2). The ODCase yield is nearly 40% in Tris or P_i buffer (Tables 1 and 2) but becomes about 50% with the addition of BSA (Table 2).

Figure 3 demonstrates directly the effect of P_i ions on the OPRTase and the ODCase activities of pure human UMP synthase using the spectrophotometric assay, where enzyme is always at a concentration high enough that dilution is not a problem. Since dilution denatures UMP synthase and since this effect is particularly dramatic for OPRTase activity when measured by the radioactive assay (Kavipurapu & Jones, 1976), the concentration of the UMP synthase is important. It is clear that P_i ions are essential for optimal OPRTase activity while P_i ion has little or no activating effect on ODCase activity. P_i can be an inhibitor of both OPRTase and ODCase activities since P_i is a competitive inhibitor vs OMP for ODCase when assayed in Tris buffer (Traut *et al.*, 1980). For OPRTase we have not determined whether P_i fills the binding site for the 5′ P of PRPP [which presumably is also the binding site for the 5′ P of OMP; see Scapin *et al.* (1994)] or whether it fills the binding site for PP_i and the pyrophosphoryl group of PRPP. However, P_i must be present for pure UMP synthase to have optimal OPRTase activity, which suggests that it affects the conformation of this protein.

UMP synthase activities of a crude homogenate or in the resuspended ammonium sulfate pellet from human placenta are not strongly affected by dilution in phosphate buffer. Presumably the presence of P_i ions in the buffer used to suspend crude extracts and ammonium sulfate pellets and high levels of other proteins in these preparations protect UMP synthase from denaturation. However, the pure protein is again labile to dilution unless it is diluted in phosphate buffer with BSA.

Occluded insect viruses may contain an endogenous alkaline (optimum pH of 9.5) protease (Summer & Smith, 1975) which is a serine protease and can be inactivated with Hg^{2+}, Cu^{2+}, and DIFP (Maruniak, 1986). In the final preparation of UMP synthase without DIFP, the expressed protein was proteolytically digested. The presence of 10 mM DIFP during the purification greatly reduced the proteolysis of UMP synthase. In the presence of the protease inhibitors listed in the experimental procedures, UMP synthase from cabbage looper larvae was purified about 80−90-fold with 40−42% yield of ODCase activity and an apparent 17% yield of OPRTase activity by the radioactive assay using Tris buffer. However, the activities of ODCase and OPRTase are so improved by the use of a buffer with phosphate plus BSA (Table 2) that the yield of pure protein is 50% regardless of which enzyme activity is measured, and the ratio of ODCase/OPRTase activities as measured by the radioactive assay (at 37 °C) is 1.8.

The use of the spectrophotometric rather than the radioactive assay also yields a more favorable OPRTase activity such that the ratio of ODCase/OPRTase is 2.3 at RT (ca. 20−22 °C). We have most confidence in the spectrophotometric values because the final UMP synthase concentrations in the assay vessels are higher (at least 10-fold for OPRTase and 100-fold for ODCase) than the concentrations of UMP synthase used for the radioactive assay. These higher concentrations may stabilize the two activities. Also, only the initial rates were used for determining the two activity values using the spectrophotometric assay. Unfortunately the spectrophotometric assays cannot be used with crude homogenates.

The common occurrence of acetylation at the N-terminus of many cytosolic proteins in eukaryotic cells has been reviewed by Driessen *et al.* (1985). The cDNA sequence of human UMP synthase determined by Suttle *et al.* (1988) predicts that the N-terminal pentapeptide is MAVAR. Tsunasawa *et al.* (1985) studied the effect of N-terminal sequences on acetylation and demonstrated that the N-terminal methionine may be acetylated or excised from the protein with subsequent acetylation of the penultimate residue. Assuming that UMP synthase is acetylated, this would produce acetyl-AVAR as the N-terminal structure.

In this report, the actual structure of the N-terminus of human UMP synthase has been determined by isolating and

[4] Lysine K314 of human UMP synthase is equivalent to the lysine K93 of yeast ODCase. The K93C analog yeast protein lacks ODCase activity, for this lysine is an essential catalyst for the decarboxylation (Smiley & Jones, 1992).

characterizing the short peptide produced by cleavage on the carboxyl side of the first arginine. The amino terminus acetyl-AVAR was found to be the form of the protein that is isolated from human tissue and this would give a protein of 479 amino acids and a calculated molecular weight of 52 133. In the analysis by mass spectrometry/CID (Figure 4), a series of fragments resulting from the peptide sequence acetyl-AVAR is present: b_1 is (acetyl-A), b_2 is (acetyl-AV), y_1 is (C-terminal R), and y_4 is (AVAR). Also, the lack of y_3 (which would be VAR, MW = 345.4) may be accounted for by further fragmentation of y_3 to w_3 and v_3 ions, which are abundant in the spectrum. Several other peaks in the spectrum may be accounted for as follows: 372.1, loss of part of the arginine side chain at the β,γ-bond; 328.9, loss of part of the arginine side chain and the complete valine side chain; and 87.3, the protonated arginine side-chain fragment resulting from fragmentation at the β,γ-bond. The predominance of y, v, and w types of fragments is consistent with previous observations for peptides containing an arginine at the C-terminus (Biemann, 1990). These results (Figure 4) show that a peptide of the expected structure (acetyl-AVAR) is obtained from both human UMP synthase expressed by recombinant baculovirus in *T. ni* larvae and UMP synthase purified from human placenta. Therefore, the N-terminal structure of human UMP synthase that was expressed in the baculovirus system is identical to that in protein obtained from human tissue.

In summary, we have constructed a recombinant baculovirus which produces a larval homogenate with 180-fold enrichment of human UMP synthase over the amount in a placental homogenate. New assay conditions have been studied and the need for P_i ions for optimal OPRTase activity was discovered. UMP synthase of high purity, 96% or 90%, can be produced from extracts of human placenta or from larval extracts, respectively, by use of a monoclonal immunoaffinity column. The baculovirus expression system allows us to easily produce, store, and process large quantities of structurally authentic human UMP synthase as well as proteins modified by recombinant DNA methods. The vector has already been used to produce a K314C analog protein[4] that lacks ODCase activity but retains OPRTase activity as well as truncated proteins having only ODCase or OPRTase activity (unpublished studies of Han, Yablonski, Pasek, Traut, and Jones).

ACKNOWLEDGMENT

We thank Rebecca Gooding for excellent technical expertise and thoughtful planning of certain experiments. We also thank Dipa Bhaumik, Ph.D., and Karen Gathy at UNC-CH for their advice on the culture of *Sf9* cells and cabbage looper larvae and for general help with the baculovirus expression. We are most grateful to Dr. David Klapper (Professor in the Department of Microbiology and Immunology) for his expert technical advice and for amino acid analysis studies which are not reported in detail but which were essential for our work. We are grateful to Dr. T. W. Traut for his advice, discussion, and suggestions for improvement of this paper.

REFERENCES

Alvarado, C. S., Livingstone, L. R., Jones, M. E., Ravielle, A., McKolanis, J., & Elsas, L. J. (1988) *J. Pediatrics 113*, 867–871.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) in *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley-Interscience, New York.

Bell, J. B., & Jones, M. E. (1991) *J. Biol. Chem. 266*, 12662–12667.

Biemann, K. (1990) *Methods Enzymol. 193*, 455–479.

Bigbee, W. L., Vanderlaan, M., Fong, S. S. N., & Jensen, R. H. (1983) *Mol. Immunol. 20*, 1353.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Drapeau, S. R. (1977) *Methods Enzymol. 47*, 189–191.

Driessen, H. P. C., de Jong, W. W., Tesser, G. I., & Bloemendal, H. (1985) *Crit. Rev. Biochem. 18*, 281–325.

Fling, S. P., & Gregerson, D. S. (1986) *Anal. Biochem. 155*, 83–88.

Floyd, E. E., & Jones, M. E. (1985) *J. Biol. Chem. 260*, 9443–9451.

Gross, E. (1967) *Methods Enzymol. 11*, 239–254.

Gurd, K. (1990) *Methods Enzymol. 25*, 424–438.

Jones, M. E. (1980) *Annu. Rev. Biochem. 49*, 253–279.

Jones, M. E., Kavipurapu, P. R., & Traut, T. W. (1978) *Methods Enzymol. 51*, 155–167.

Kavipurapu, P. R., & Jones, M. E. (1976) *J. Biol. Chem. 251*, 5589–5599.

Kirschenbaum, D. M. (1973) *Anal. Biochem. 55*, 166–192.

Konigsberg, W. (1972) *Methods Enzymol. 25*, 185–188.

Kunkle, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Livingstone, L. R. (1984) *Diss. Abstr. 45*, 1177-B.

Livingstone, L. R., & Jones, M. E. (1987) *J. Biol. Chem. 262*, 15726–17733.

Luckow, V. A. (1991) in *Recombinant DNA Technology and Applications* (Prokop, A., Bajpai, K., & Ho, C., Eds.) pp 97–152, McGraw-Hill, New York.

Maruniak, J. E. (1986) in *The Biology of Baculoviruses* (Granados, R. R., & Federici, B.A., Eds.) Vol. 1, p 133, CRC Press, Inc., Boca Raton, FL.

McClard, R. W., Black, M. J., Livingstone, R. L., & Jones, M. E. (1980) *Biochemistry 19*, 4699–4706.

Medin, J. A., Hunt, L., Gathy, K., Evans, R. K., & Coleman, M. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2760–2764.

Perry, M. E., & Jones, M. E. (1989) *J. Biol. Chem. 264*, 15522–15528.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Scapin, G., Grubmeyer, C., & Sacchettini, J. C. (1994) *Biochemistry 33*, 1287–1294.

Smiley, J. A., & Jones, M. E. (1992) *Biochemistry 31*, 12162–12168.

Silva, R. F., & Hatfield, D. (1978) *Methods Enzymol. 51*, 143–154.

Suttle, D. P., Bugg, B. Y., Winkler, J. K., & Kanals, J. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1754–1758.

Summer, M. D., & Smith, G. E. (1975) *J. Virol. 16*, 1108–1116.

Summer, M. D., & Smith, G. E. (1987) *A manual of methods for baculovirus vectors and insect cell culture procedures*, Bulletin 155, Texas Agricultural Experimental Station and Texas A&M University, College Station, TX.

Traut, T. W., Payne, R. C., & Jones, M. E. (1980) *Biochemistry 19*, 6068–6074.

Tsunasawa, S., Stewart, J. W., & Sherman, F. (1985) *J. Biol. Chem. 193*, 5382–5391.

Walther, R., Wald, K., Glund, K., & Tewes, A. (1984) *J. Plant Physiol. 116*, 301–311.

BI950510J