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Intrinsic Activity and Stability of Bifunctional Human UMP Synthase and Its Two Separate Catalytic Domains, Orotate Phosphoribosyltransferase and Orotidine-5′-phosphate Decarboxylase*

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Human UMP synthase is a bifunctional protein containing two separate catalytic domains, orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (EC 4.1.1.23). These studies address the question of why the last two reactions in pyrimidine nucleotide synthesis are catalyzed by a bifunctional enzyme in mammalian cells, but by two separate enzymes in microorganisms. From existing data on subunit associations of the respective enzymes and calculations showing the molar concentration of enzyme to be far lower in mammalian cells than in microorganisms, we hypothesize that the covalent union in UMP synthase stabilizes the domains containing the respective catalytic centers. Evidence supporting this hypothesis comes from studies of stability of enzyme activity *in vitro*, at physiological concentrations, of UMP synthase, the two isolated catalytic domains prepared by site-directed mutagenesis of UMP synthase, and the yeast ODCase. The two engineered domains have activities very similar to the native UMP synthase, but unlike the bifunctional protein, the domains are quite unstable under conditions promoting the dissociated monomer.

The final two steps in the *de novo* biosynthesis of UMP require the addition of ribose-P to the pyrimidine base orotate by orotate phosphoribosyltransferase (OPRTase)[1] to form OMP and the subsequent decarboxylation of OMP to form UMP by orotidylate decarboxylase (ODCase). In all microorganisms examined, these two catalytic centers are coded by two separate genes, while in all multicellular eukaryotes examined, the genes for these two catalytic centers have been joined into a single gene, resulting in the expression of the bifunctional protein, UMP synthase, with two different catalytic domains (1) (Fig. 1*A*).

Monomeric UMP synthase can be converted to a simple dimer, with an $s_{20,w}$ of 5.1, by various anions such as phosphate, and to a faster sedimenting 5.6 S dimer with optimal activity by the normal ligand, OMP, or by nucleotide analogs (2, 3) (Fig. 1*B*). An important feature of this model is that there are two conformational states of the dimer, based on sedimentation studies showing that only nucleotides produced the more rapidly sedimenting 5.6 S species (2, 3), on studies showing that a different type of tryptic digestion pattern for the pure enzyme was obtained in the presence of these tight binding nucleotides, than in the presence of simple anions (4), and on studies showing that enzyme preincubated to be in the 5.6 S form had optimum activity immediately after the addition of substrate, while enzyme preincubated in the 5.1 S form had a 20-s lag time after substrate addition before attaining optimum activity (5). Such conformational features have been found for a large set of dissociating enzymes, whose diagnostic feature is the reversible interconversion between monomeric and oligomeric forms, which occurs in response to physiological concentrations of appropriate regulatory effectors (6).

Three different important benefits are likely candidates for the evolution of the bifunctional architecture: 1) the two catalytic centers may interact to "channel" the intermediate OMP; 2) an element of allosteric control is jointly communicated between the two different domains; 3) the intact bifunctional protein is more stable than the separated catalytic domains. Channeling of intermediate metabolites between catalytic centers remains a controversial topic as shown in a recent symposium (7), with channeling being supported with some enzymes but not for others. Our earlier studies had tested the channeling hypothesis for UMP synthase with mouse cell extracts, which would contain normal quantities of enzymes that might compete for any OMP being formed by the OPRTase domain of UMP synthase (8). These studies showed that channeling was not efficient, since part of the OMP made by UMP synthase was readily converted to orotidine by an available phosphatase activity, thereby verifying that some OMP had to have diffused into the bulk solvent during the assay.

There is better support for the other two benefits, since the dimerization of UMP synthase in response to effectors results in better activity for each catalytic domain as suggested in Fig. 1 (2, 3, 5, 9). Also, such an architecture could serve to enhance the stability of the enzyme as a monomer or as a dimer. This latter benefit was suggested by the observation described in the text that the intracellular concentrations of monofunctional OPRTase and ODCase in microorganisms are 10–100-fold the concentration of the bifunctional UMP synthase in mammalian cells and was verified by the experiments described.

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[1] The abbreviations used are: OPRTase, orotate phosphoribosyltransferase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ODCase, orotidine 5′-monophosphate decarboxylase; OMP, orotidine-5′-phosphate.

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EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated sources: sodium [7-^{14}C]orotate and sodium [7-^{14}C]OMP (DuPont NEN); polyethylene glycol, molecular weight 10,000 (Fluka); dithiothreitol, ultrapure (Boehringer Mannheim); 98–99% pure bovine serum albumin (Sigma). 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 (10). All other chemicals were reagent grade or as specified in the appropriate references.

Construction and Expression of Plasmids—Plasmid pAcUMPS containing the coding region for UMP synthase (11) was used as a polymerase chain reaction template for construction of plasmids. With the OPRTase domain at the 5′-end, the plasmid was cut at a unique *Nhe*I site 57 codons into the downstream region of the ODCase domain. These extra codons of the ODCase domain were removed by a loop-out mutagenesis using a primer which placed the stop codon immediately after the codon for glycine 213 of the OPRTase domain in plasmid pAcOPRT. Mutagenesis was according to the method of Kunkel (12) utilizing the single-stranded DNA produced by phage M13mp19.

Plasmid pAcODC was constructed by making a polymerase chain reaction copy of the ODCase coding region beginning at isoleucine 218. A primer was designed to change isoleucine to methionine while incorporating the methionine codon into a new *Nco*I site. The polymerase chain reaction product was trimmed with *Nco*I and ligated into *Nco*I cut pAcUMPS. Plasmids of the correct sequence were identified by restriction analysis and confirmed by sequencing the DNA across the N-terminal junction. Recombinant plasmids were grown in *Escherichia coli* and purified by CsCl gradient centrifugation in preparation for the production of recombinant baculoviruses. Thereafter, cabbage looper larvae were injected with recombinant baculovirus as reported previously (11). After injection into the larvae, the expression of the proteins was followed by Western blots and activity assays with larval extracts, and optimum expression occurred at 5 days post-infection.

Purification of Expressed UMP Synthase and the Two Domains—The expressed UMP synthase, K314C variant, and ODCase domain were purified on a monoclonal antibody affinity column (11). Homogenates of cabbage looper larvae producing each protein were made using the same protocol except that the larvae were homogenized in the standard buffer, adjusted to pH 6.8 (at 0 °C) and containing 25% ammonium sulfate. The ODCase domain and OPRTase domain were precipitated at 65% ammonium sulfate saturation. After storage of the ammonium sulfate pellet, OPRTase was further purified by specific elution from a phosphocellulose column. The sample was diluted 25-fold in buffer (20 mM MOPS, 1 mM EDTA, pH 6.8) and loaded onto the column (10 ml). The column was then washed with 100 ml of buffer, 50 ml of buffer containing 0.05 M KCl followed by 50 ml of buffer, and the last two washes were repeated until the absorbance at 280 nm was unchanged. The OPRTase domain was then eluted with 50 ml of buffer containing 0.04 M KCl and 0.1 mM OMP. Elution fractions were concentrated in an Amicon ultrafiltration cell to a volume of about 8 ml, then in a collodion bag for about 5 h *versus* collodion dialysis buffer 2 (11). Protein concentrations were determined by the method of Bradford (13).

Enzyme Assays—ODCase activity was determined at 25 or 37 °C by measuring the release of $^{14}CO_2$ from [^{14}C]OMP as described previously (14) or by a spectrophotometric assay measuring the decrease in absorbance at 285 nm for the conversion of OMP to UMP (11). OPRTase activity was determined at 25 or 37 °C by measuring the release of $^{14}CO_2$ from [^{14}C]orotate after sequential conversion to OMP and UMP + $^{14}CO_2$ or by a spectrophotometric assay measuring the decrease in absorbance at 295 nm for the conversion of orotate to OMP and to UMP (11). Changes to these standard protocols are given under Results or in figure legends. For assays of the OPRTase domain in the forward reaction, an equimolar amount of the human ODCase domain was added to ensure conversion of OMP to UMP + 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 the substrate. Enzyme dilutions were made prior to use in the standard buffer.

Enzyme activity for the Arrhenius plots was measured spectrophotometrically, with the temperature controlled by a thermo-electric cuvette holder. Enzyme was added at a concentration of 10–15 μg/ml, and substrate concentrations were at 100 μM orotate plus 0.3 mM P-Rib-PP for the forward OPRTase reaction or at 100 μM OMP plus 1.2 mM PP_i for the reverse OPRTase activity or at 200 μM OMP for ODCase activity.

Sucrose Density Gradient Centrifugation—Experiments were generally performed as described previously (15). Gradients (10 ml of a 10–40% sucrose solution containing 10% glycerol) were made in standard buffer: 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM dithiothreitol. Gradients were layered with a 200-μl enzyme sample containing enzyme protein, plus M_r standards. Many gradients also contained effector ligands at specified concentrations, based on the previously determined affinity of such ligands for the enzyme.

TABLE I
Specific activities and substrate affinities at 37 °C for the bifunctional UMP synthase and the separate catalytic domains

Enzyme species	k	Substrate: K_m
	s^{-1}	
OPRTase-bifunctional:	4.0	Orotate: 2.1 ± 0.12 μM
OPRTase domain:	2.9	Orotate: 7.1 ± 0.27 μM
ODCase-bifunctional:	16	OMP: 230 ± 8.7 nM
ODCase domain:	13	OMP: 295 ± 18 nM

Calculations—Estimates of the concentration *in vivo* of the bifunctional UMP synthase or of the monofunctional ODCase and OPRTase were obtained from published purifications, to provide the protein concentration, c (nM),

$$c = \frac{(\text{total activity}_{\text{extract}}/\text{SA}_{\text{pure}})}{(\text{g total tissue} \times 0.8 \text{ ml/g})/(M_r \times 10^{-6})} \quad \text{(Eq. 1)}$$

where SA is the specific activity of pure enzyme, and the cytoplasmic volume of tissue is approximately 0.8 ml/g for three different mammalian tissues (16–18).

RESULTS AND DISCUSSION

Preparing the Separate Domains—The current data base shows the consensus sequence for 16 monofunctional OPRTases from bacteria and fungi does not extend beyond residue 208 of UMP synthase (480 amino acids total), while a sequence alignment with 31 monofunctional ODCases suggests that the consenus structure for the ODCase domain begins after amino acid 224. To ensure producing complete domains, we engineered a plasmid with the sequence for the human UMP synthase to produce truncated cDNAs for the OPRTase domain ending at residue 214 and for the ODCase domain beginning at residue 218. Recombinant baculoviruses for the complete UMP synthase and for both the OPRTase and ODCase domain expressed proteins of the expected size, and these domains were specifically recognized in a Western blot by a polyclonal antibody raised against human UMP synthase (data not shown).

Activities—When these protein domains were purified, they showed enzymatic activity very similar to that observed with the bifunctional protein. These are the first studies that have determined both the absolute rates and the K_m values for each catalytic center with the bifunctional UMP synthase, as well as with the separated domains (Table I). For the OPRTase, the K_m for orotate with the bifunctional human UMP synthase is 2.1 μM, a value almost the same as measured with the same enzyme from mouse (3). The OPRTase domain has a somewhat higher K_m of 7.1 μM.

The human ODCase has a K_m of 230 nM for OMP, a value very similar to earlier measurements for the mammalian enzyme (5, 19). By comparison the human ODCase domain had a slightly higher K_m of 295 nM. These results verify that the domains, produced by our changes of the human UMP synthase cDNA, behave as if they are properly folded proteins with normal enzymatic rates and affinities for substrates.

Dimerization of UMP Synthase—Since earlier studies with the mammalian UMP synthase and the yeast ODCase had demonstrated the allosteric response of these enzymes to effector ligands as measured by the association of monomers to form the dimer (2, 3, 10, 15), sedimentation studies were done with the pure human UMP synthase. This conformational response to allosteric ligands was readily measured with the pure enzyme: UMP synthase at concentrations below 15 nM was very stable as a monomer, and it generally took enzyme concentra-

TABLE II
Estimated concentrations in vivo of UMP synthase (UMPS) and the monofunctional OPRTase and ODCase

Cell/tissue	Concentration			Ref.
	[UMPS]	[OPRT]	[ODC]	
		nM		
Mammal:				
Human placenta	17			19
Human lymphocytes	32			27
Rat liver	11			28
Rat brain	27			29
Microorganisms:				
E. coli			2900	26
E. coli		2720		24
Yeast		950	260	22
Yeast			375	30

tions ≥ 100 nM to produce association to the dimer in the absence of an effector nucleotide ligand (data not shown). However, at enzyme concentrations of about 10 nM, the presence of various high affinity nucleotides produced the conformational change that stabilized the dimer form. The tight binding inhibitor nucleotide barbiturate riboside monophosphate (K_i = 4.1 nM) produced complete dimerization at a concentration equal to the enzyme.

Dependence of Catalytic Rates on Temperature—The effect of temperature on OPRTase and ODCase catalytic activities was tested with enzyme at concentrations of 230–640 nM and substrates at 100 μM. These optimal concentrations are far above the physiological concentrations for the enzyme (Table II), or the K_m values determined for their substrates (Table I). The measured enthalpy was ΔH = 9.7 kcal/mol for the forward OPRTase reaction (orotate → OMP) when measured with UMP synthase, or with the separated OPRTase domain, and ΔH = 21.7 kcal/mol for the reverse OPRTase reaction (OMP → orotate). This difference in the intrinsic energy for this reaction, depending on direction, is completely consistent with earlier results showing that the reverse reaction is kinetically more favorable than the forward reaction (3). For the ODCase reaction ΔH = 14.9 kcal/mol when measured with UMP synthase or with the separated ODCase domain. These temperature studies for both reactions also demonstrate that under optimized experimental conditions there is no difference in the observed activity for the reaction as measured with the bifunctional UMP synthase and the separate OPRTase or ODCase domain, signifying that under these conditions the isolated proteins are stable and completely active at the temperatures used, and for the times used.

Thermal Stability of the Two Catalytic Centers—Since the above experiments were done under conditions that are clearly not completely physiological, additional studies were done to properly assess the bifunctional protein and the two separate domains under conditions that could explore their stability as a function of protein concentration, which influences whether the enzyme is a monomer or a dimer. ODCase activity could be reliably measured at about 10 pM. At this low concentration of enzyme, it was also possible to use an initial substrate concentration near K_m, so that the enzyme would not be saturated with bound substrate and thus increase the detection of instability since part of the enzyme population would remain unbound to ligand and therefore in the monomeric form at all times (Fig. 1). To compare the different progress curves, data are therefore presented as the percent substrate converted to product.

The ODCase activity of the bifunctional UMP synthase at this low protein concentration remains constant for 40 min at 25 °C (Fig. 2*A*), and at 37 °C declines very slightly after about 15 min when more than 10% of the substrate has been depleted. At this low concentration of enzyme, the ODCase domain is much less stable, and the progress curve becomes nonlinear after only 2 min at either 25 °C or at 37 °C, before any significant depletion of substrate has occurred (Fig. 2*B*). While the ODCase domain and the bifunctional ODCase have inherently similar activities under optimal conditions where the enzymes are dimeric, in the experiment of Fig. 2 it is readily apparent that when the ODCase domain is diluted enough to keep the enzyme partly monomeric, its intrinsic activity is the same as the activity in UMP synthase for about the first 2 min, whereafter it becomes much lower than that of the UMP synthase.

The yeast ODCase is even more unstable than the human ODCase domain, and activity declined after only 1 min, and the decline in activity at 37 °C was more dramatic (Fig. 3). Also, the yeast ODCase showed much lower total activity at 37 °C, consistent with increased protein instability at this temperature.

Similar stability experiments were done with the OPRTase reaction, but with protein at a concentration of 100 pM to ensure reproducible measurements. For the OPRTase in UMP

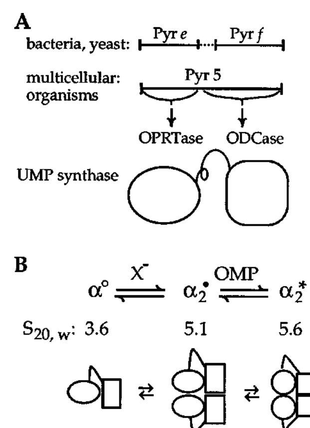

FIG. 1. **Genes and possible structures for UMP synthase.** *A*, natural gene fusion led to the fusion of catalytic domains. *B*, conformational states: phosphate and anionic analogs promote association of inactive subunits to the simple dimer, as measured by sedimentation. OMP, or nucleotide analogs, produce an additional conformational change, measured by increased sedimentation and necessary for optimum enzyme activity. ○ = inactive; ● = partly active; * = active.

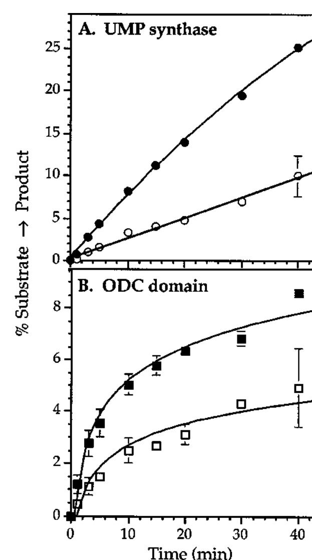

FIG. 2. **Stability of the dilute human ODCase catalytic center.** Enzyme at a final concentration of 10.5 pM was incubated for the time shown with the substrate OMP at 230 nM, at 37 °C (*closed symbols*) or at 25 °C (*open symbols*).

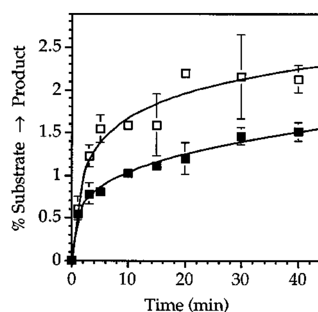

FIG. 3. **Stability of dilute yeast ODCase.** Enzyme at a final concentration of 10.1 pM was incubated with the substrate OMP at 700 nM, at 37 °C (■) or at 25 °C (□).

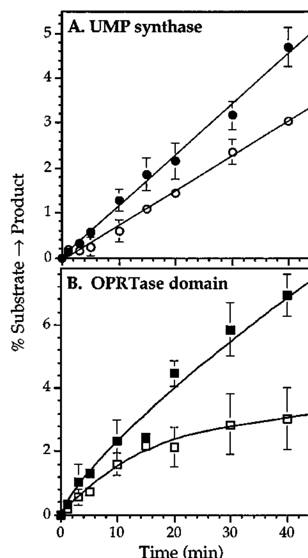

FIG. 4. **Stability of the dilute human OPRTase catalytic center.** Enzyme at a final concentration of 100 pM (*A*) or at 1.0 nM (*B*) was incubated for the time shown with the substrate orotate at 2.0 μM, at 37 °C (*closed symbols*) or at 25 °C (*open symbols*).

synthase, enzyme activity remained linear and stable at both temperatures and for the full 40-min time period (Fig. 4*A*). Since our initial studies showed that the OPRTase domain was a little less active under these experimental conditions, it was assayed with the domain at a concentration of 1.0 nM. Though at a 10-fold higher concentration than UMP synthase, the OPRTase domain's activity was not linear at 37 °C or at 25 °C (Fig. 4*B*). While the diluted ODCase domain appeared to be equally unstable at 37 °C or at 25 °C (Fig. 2*B*), the OPRTase domain appeared more unstable at the lower temperature (Fig. 4*B*).

To test these activities at physiological concentrations of enzyme, an effort was made to estimate what these concentrations might be, by using data from published purification results (see "Experimental Procedures"). It is apparent that in four normal mammalian tissues, the bifunctional UMP synthase has cellular concentrations estimated to be in the 11–32 nM range (Table II). By comparison, mitotically active tumor cells have increased the concentration of this important bifunctional protein by about 10-fold or more (20, 21), while microorganisms have concentrations of the separate catalytic enzymes that are very much higher (Table II). Therefore, additional stability experiments were done with enzyme concentrations at 40 nM, a value at the upper end of normal physiological concentrations in mammals.

The stability experiment at these higher enzyme concentrations had to be modified, since activity cannot be maintained

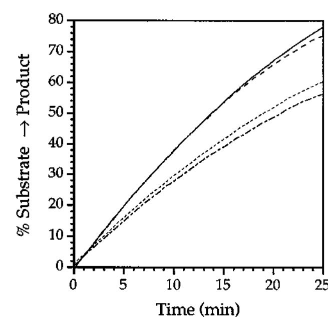

FIG. 5. **Stability of concentrated ODCase at 25 °C.** Enzyme at a final concentration of 40 nM was initially preincubated for 1 min using UMP synthase (——) or ODCase domain (— — — —), or for 40 min. with UMP synthase (— — — — —) or ODCase domain (– – – –). After the preincubation period, enzyme activity was then initiated by the addition at 0 min of the substrate OMP at 200 μM.

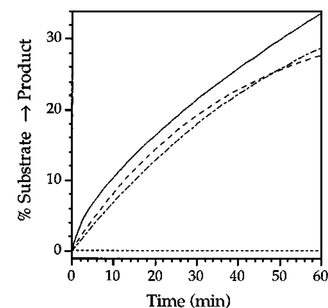

FIG. 6. **Stability of concentrated OPRTase at 25 °C.** Enzyme at a final concentration of 40 nM was initially preincubated for 1 min using UMP synthase (——) or OPRTase domain (— — — —) or for 40 min with UMP synthase (— — — –) or OPRTase domain (– – – –). Activity was then initiated by the addition at 0 min with the substrate orotate at 100 μM and P-Rib-PP at 300 μM.

for such long time periods (40 min) at low substrate concentrations. Therefore, enzyme at a final concentration of about 40 nM was preincubated in buffer for either one minute, or for 40 min, at which time substrate was added to a concentration ≥ 50 × K_m, and continuous activity was monitored after the addition of substrate at 0 min to the preincubated enzyme (Fig. 5). When either UMP synthase or the human ODCase domain was preincubated for 1 min, there was no detectable change in enzyme activity: the progress curves were linear until more than 30% of the substrate had been depleted. However, when the enzyme samples had been preincubated at 25 °C for 40 min in the absence of any ligands, there was about a 20% decline in the rate of the initial progress curve, and this decline was comparable for the bifunctional UMP synthase and for the ODCase domain.

The OPRTase domain, by comparison, was more unstable even at these higher concentrations (40 nM). The 1-min preincubation had no dramatic effect on the activity, and the curvature evident in Fig. 6 reflects the sensitivity of this assay to the accumulation of products and the possibility of the back reaction. However, when these same enzyme samples had been preincubated for 40 min, the bifunctional protein showed a modest decrease in the initial rate of activity, while the separate OPRTase domain had no detectable activity when maintained at 25 °C for 40 min in the absence of any ligands. This dramatic loss of activity was very reproducible. Separate experiments (not shown) gave no evidence for proteolysis producing the total loss of enzyme activity seen for the OPRTase in Fig. 6.

Although earlier studies with UMP synthase had noted the instability of the OPRTase activity, this difficulty was resolved by optimizing the storage and assay buffers as recently de-

scribed (11). This improved stability of the OPRTase activity in the bifunctional native protein is now evident in Fig. 4*A*.

In the experiments of Figs. 2–4, enzyme was used at a very low concentration to ensure that the protein would be monomeric in the absence of ligands, and the initial concentration of the substrate, OMP or orotate, was near K_m so that a significant fraction of the enzyme population would remain ligand-free at any time and therefore be in the monomeric state. Under these conditions, as a function of time in the assay, enzyme molecules should alternate between the monomer and active dimer states (Fig. 1). As shown by the results of Figs. 2*B* and 3, the isolated human ODCase domain, as well as the yeast ODCase monofunctional protein, were very unstable under conditions that favored the monomeric state. Fig. 4*B* shows a comparable instability for the OPRTase domain, at a concentration where it would be expected to be monomeric for part of the assay time. Thus, the data of Figs. 2*A* and 4*A* are remarkable in demonstrating the complete stability of each catalytic center when it is in the bifunctional architecture, even when UMP synthase was partly in the monomeric form. A possible explanation for the evident stability of the ODCase and OPRTase centers of the bifunctional UMP synthase is that there may be interaction between the two different domains within the same subunit, as modeled in Fig. 1. Even if such interaction between the OPRTase and ODCase domains is transient, it could add sufficient stabilization to maintain the active structural conformation of each domain during the time that the protein subunit is monomeric, so that there is no measurable loss of activity. By comparison, both the separate human ODCase domain, the yeast ODCase, and the separate human OPRTase domain were unstable at concentrations expected to favor the monomeric state, as measured by dramatic loss of activity in only a few minutes under otherwise benign conditions.

It is clear that the independent forms of OPRTase and ODCase in bacteria and yeast must function successfully, so that the instability observed here was more pronounced due to the experimental conditions. However, the lower cellular concentration for the mammalian UMP synthase (Table II) is not a simple benefit of UMP synthase having more efficient catalytic centers. The opposite is true, since both OPRTases and ODCases from microorganisms have intrinsic activities that may be up to 6 or 8 times as fast as the UMP synthase values in Table I. Calculated values for k_{cat} of OPRTase from bacteria or yeast are 12–32 s^{-1} (22–25), and the values for ODCase from microorganisms are 37–108 s^{-1} (10, 26). Our studies show that UMP synthase becomes a dimer at an enzyme concentration ≥ 100 nM, while at a concentration of 750 nM yeast ODCase remains monomeric (data not shown). Thus, even though they are catalytically more efficient, the much larger cellular concentration for these enzymes in microorganisms may in part be necessary for stability. At such cellular concentrations, the monofunctioal OPRTase and ODCase enzymes would be predominantly or totally in the more stable and active dimeric conformation. By comparison, the bifunctional UMP synthase does not need to be dimeric for stability, since interaction between domains within a subunit may serve a similar function, such that both catalytic centers of UMP synthase remain stable under conditions where the separated domains lose activity.

In addition to the present studies demonstrating that stability is a benefit of the bifunctional architecture, the extent to which OMP channeling may occur remains to be explored. While our earlier studies with cell extracts showed that channeling of OMP was incomplete (8), with the availability of pure UMP synthase and the two domains, future studies will compare the efficiency of OMP transfer within the bifunctional enzyme in comparison to a mixture of the two domains.

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