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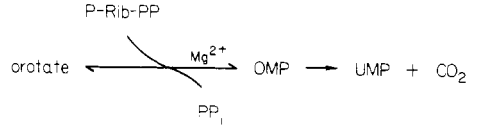
Dependence of the Catalytic Activities on the Aggregation and Conformation States of Uridine 5′-Phosphate Synthase†

Thomas W. Traut* and Robert C. Payne

ABSTRACT: Uridine 5′-phosphate (UMP) synthase is a multifunctional protein that contains the last two enzyme activities for the de novo biosynthesis of UMP, orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidine-5′-phosphate (OMP) decarboxylase (EC 4.1.1.23). The native enzyme from mouse Ehrlich ascites cells exists in at least three distinct aggregation/conformation states as measured by sedimentation in sucrose gradients: a 3.6S monomer, a 5.1S dimer, and a conformationally altered 5.6S dimer. It has previously been reported that a variety of ligands (of which the most effective is OMP) mediate the conversion of the 3.6S monomer to the two types of dimers. Initial velocity studies with the enzyme in the different native states show that all three forms of UMP synthase have phosphoribosyltransferase activity but that the OMP decarboxylase is either uniquely or at least predominantly associated with the 5.6S form. Activation of this enzyme activity by the substrate appears to be the result of both a dimerization and a conformation step.

The two enzyme activities of UMP synthase,[1] orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidine-5′-phosphate (OMP) decarboxylase, catalyze the last two enzymatic steps of de novo UMP biosynthesis.



Previous studies have shown that native UMP synthase exists in at least three different physical states, with sedimentation constants of 3.6 for the monomer and 5.1 or 5.6 for the dimer (Traut & Jones, 1979; Traut et al., 1980). The aggregation of the 3.6S monomer to the 5.1S dimer is mediated by a variety of effectors [nucleoside monophosphates, 5-phosphoribosyl 1-pyrophosphate (P-Rib-PP), P_i, etc.] binding to the OMP decarboxylase catalytic site (Traut & Jones, 1979). A conformational alteration of the 5.1S dimer to the 5.6S dimer is produced only by pyrimidine nucleotides acting on a regulatory site that appears to be distinct from either of the two catalytic sites (Traut et al., 1980). The formation of the 5.6S species of UMP synthase is promoted most effectively by OMP, the product of one catalytic center, and the substrate for the second catalytic site of UMP synthase. Kinetic studies suggest that the aggregation/conformation state produced by the substrate OMP increases the catalytic activity of the OMP decarboxylase.

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[1] Previous publications on UMP synthase have used the designation "complex U" for the single polypeptide chain which possesses these two enzyme activities. Because of the recent purification of a single homogeneous polypeptide chain with both enzyme activities, the term "complex U" has been replaced by "UMP synthase". A more precise term has also been suggested, "multienzyme *pyr 5,6*" (Jones, 1980; Christopherson et al., 1980).

0006-2960/80/0419-6068$01.00/0

Experimental Procedures

Materials

The following materials were obtained from specified vendors: tetrasodium P-Rib-PP, dithiothreitol, nucleotides, and Tris base from Sigma; [6-^{14}C]orotate and [7-^{14}C]OMP from New England Nuclear; [5-^{3}H]orotate from Amersham. Poly(ethylenimine)–cellulose (PEI–cellulose) plates were from Brinkmann.

Methods

Enzyme Preparation. UMP synthase was prepared from mouse Ehrlich ascites cells as described previously (Jones et al., 1978). In the present work, the protein fraction precipitating between 1.7 and 2.6 M ammonium sulfate was used. This fraction is purified 8–10-fold.

Enzyme Assay. Orotate phosphoribosyltransferase activity was measured in a final reaction volume of 50 μL containing 20 mM Tris-HCl (pH 7.4 at 0 °C), 2 mM dithiothreitol, 5 mM $MgCl_2$, and 0.1 mM EDTA. UMP synthase, in amounts described in the figure legends, was preincubated for 20 min in the above reaction mixture with or without P-Rib-PP at 300 μM. Under these conditions, UMP synthase would exist predominantly as the 5.1S dimer (with P-Rib-PP) or as the 3.6S monomer (without P-Rib-PP). To produce the 5.6S form, OMP (final concentration at 1 μM) was added at the end of the 20-min preincubation period for an additional 30 s. The measurement of enzyme activity was initiated by the addition of [5-^{3}H]orotate (2 Ci/mmol) to a final concentration of 25 μM. When P-Rib-PP was omitted from the preincubation mixture, it was added with the orotate. All additions were made with Hamilton microliter syringes. The reaction was stopped by the addition of 10 μL of 0.4 M $HClO_4$. The above steps were all performed in a cold room with an ice–water bath.

The quenched reaction mixtures were centrifuged in an Eppendorf microcentrifuge to pellet the precipitated protein. The supernatant fractions were transferred to clean 400-μL centrifuge tubes and neutralized with an alkaline bicarbonate solution. For proper resolution of substrate and products by thin-layer chromatography, it was necessary to reduce the protein and salt contents of the samples.

For the removal of protein, 20 μL of phenol was added to each sample, and the contents were mixed vigorously. After centrifugation, the aqueous phase was sufficiently low in protein. Control tests showed that 85–95% of the radioactively labeled substrate and products was routinely recovered in the aqueous phase. An aliquot of the deproteinized sample was spotted on a PEI–cellulose plate and dried under hot air. After application of samples, the entire PEI–cellulose plate was immersed for 10 min in absolute methanol to remove most of the salts from the sample spots.[2] No radioactivity was lost by the methanol wash, and this procedure was far superior to other efforts to desalt samples prior to their application to the PEI–cellulose chromatogram. Chromatograms were developed by ascending chromatography with 0.187 M LiCl, as described elsewhere (Reyes & Guganig, 1975), and processed by a modified procedure (Jones et al., 1978).

For the experiments of Figures 1B and 7A, the phosphoribosyltransferase activity was determined in a coupled assay that measures the formation of $^{14}CO_2$ from [7-^{14}C]orotate (2 Ci/mol) by methods previously described (Jones et al., 1978).

OMP decarboxylase activity was measured in a final volume of 250 μL, containing buffer, dithiothreitol, $MgCl_2$, and EDTA as above. An aliquot of stock UMP synthase, in amounts described in the text and in figure legends, was added to the above reaction mixture and preincubated for various times as indicated. In some experiments, OMP (final concentration at 1 μM) was added for an additional 30 s after the regular preincubation period, and enzyme activity was initiated by the addition of [7-^{14}C]OMP (4 Ci/mol) to a final concentration of 25 μM. Reactions were stopped by the addition of 100 μL of 4 M $HClO_4$. All steps were carried out at 0 °C, unless indicated otherwise.

The OMP decarboxylase activity is measured by the production of $^{14}CO_2$. Reactions are therefore performed in standard scintillation vials (with an inserted glass center well for the reaction mixture) sealed with a rubber septum stopper. A 16-gauge syringe needle was inserted into the stopper to act as a guide shaft for the needle of the Hamilton syringe which was used to start the assay by the addition of enzyme or substrate. A separate syringe containing the $HClO_4$ was positioned above the reaction vessel, with its needle piercing the rubber septum just above the center well. Thus, the reaction was initiated with one syringe and could be quickly stopped with the second. Immediately after quenching the reaction with acid, the guide shaft and the acid-containing syringe were removed. Samples were then processed as described (Jones et al., 1978). These procedures were used for the experiments described in Figures 2–5.

For the experiments in Figure 7B, OMP decarboxylase activity was measured at 37 °C in a final volume of 500 μL containing the standard buffer, dithiothreitol, and [7-^{14}C]OMP at concentrations as indicated. Reactions were started by the addition of UMP synthase at concentrations defined in the figure legend and stopped after 10 min.

Storage Stability. UMP synthase at a final concentration of 6 mg/mL was stored at 4 °C in standard buffer, or buffer plus effectors as indicated. For each condition, there were two sample tubes, and aliquots were removed for assay of the two catalytic activities at varying times as indicated.

Results

Kinetic Studies with UMP Synthase at 0 °C. A variety of sedimentation studies showed that in the presence of substrates for the phosphoribosyltransferase activity about 80–90% of UMP synthase is in the 5.1S dimer form, and in the presence of OMP, the substrate for the decarboxylase activity, all of UMP synthase exists as the 5.6S dimer (Traut & Jones, 1977, 1979; Traut et al., 1980). It has also been demonstrated that UMP synthase is catalytically active in such sedimentation experiments (Traut & Jones, 1979). However, it is difficult to measure the enzymatic activity of the 3.6S monomer (this is the normal form of UMP synthase in the absence of effectors) since the addition of substrates leads to dimerization. While we cannot be sure of the time required for the monomer–dimer transition, measurements of enzyme activity at 37 °C always showed linear progress curves. Since the velocity of UMP synthase activity at 0 °C is about 5% of the velocity at 37 °C, we made use of the 20-fold increase in our time scale that was possible with kinetic measurements at 0 °C.

An Arrhenius plot of OMP decarboxylase is linear between 37 and 0 °C (Figure 1A), with an activation energy of 6.6 kcal/mol. This suggests that except for the obvious change in velocity measurements of enzyme activity at 0 °C should give results quite similar to those at 37 °C. A similar plot for the orotate phosphoribosyltransferase activity (Figure 1B) shows one break at about 7 °C, with an activation energy 12.8 kcal below 7 °C and 6.2 kcal above 7 °C. The significance of this break in the curve will be considered later.

[2] We are grateful to Dr. Richard Christopherson for suggesting this step.

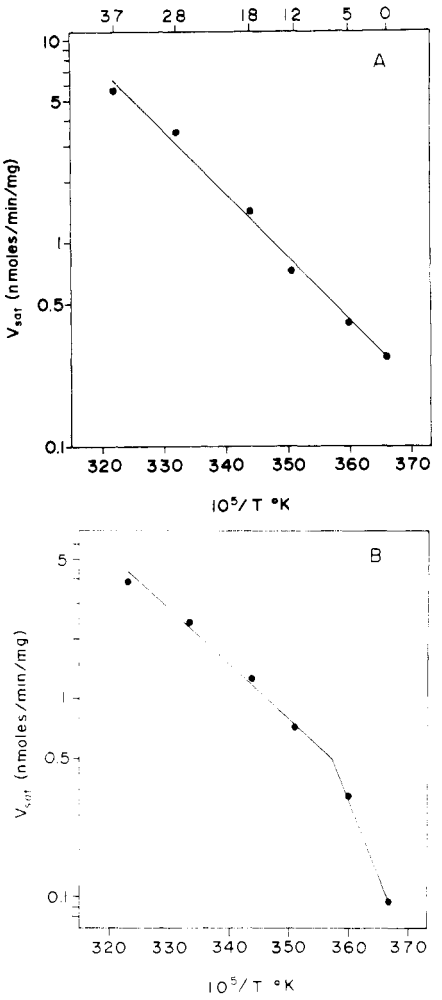

FIGURE 1: Arrhenius plot for the two UMP synthase activities at temperatures from 0 to 37 °C. Enzyme activities were measured as described under Experimental Procedures, with UMP synthase at 0.38 mg of protein/mL. Since the pH of Tris buffer is temperature dependent, separate buffer solutions were standardized to pH 7.4 at each temperature. (A) OMP decarboxylase; (B) Orotate phosphoribosyltransferase.

If UMP synthase is placed in Tris–dithiothreitol buffer (in the absence of any effector), it should all be in the form of the 3.6S monomer. Addition of the substrate OMP to such a reaction mixture will initiate a transition of the 3.6S monomer to the 5.6S dimer as well as OMP decarboxylase activity. As shown in Figure 2, there is a measurable lag time in the progress curve for the decarboxylase activity. The longer the enzyme is preincubated, the greater is the observed lag before enzyme activity becomes linear, although the observed lag time appears to have an upper limit (Figure 2 inset). For a preincubation period of 20 min, the time required for full OMP decarboxylase activity is about 6 s. Also, if a constant preincubation period is used, with UMP synthase at different concentrations, there is an increase in the lag time as enzyme concentration decreases (Figure 3).

If UMP synthase is preincubated for 20 min in the 3.6S form, then converted to the 5.6S form by an extra 30-s preincubation with unlabeled OMP (1.0 μM) before measuring enzyme activity with radioactive OMP, the progress curve is absolutely linear and passes through the origin (Figure 4); that is, when UMP synthase is in the 5.6S form, the OMP decarboxylase activity is instantaneously active. Also, there is no change in the specific activity as a function of enzyme concentration. This experiment demonstrates that the lag times shown in Figures 2 and 3 are not an artifact resulting from poor mixing or some methodological error.

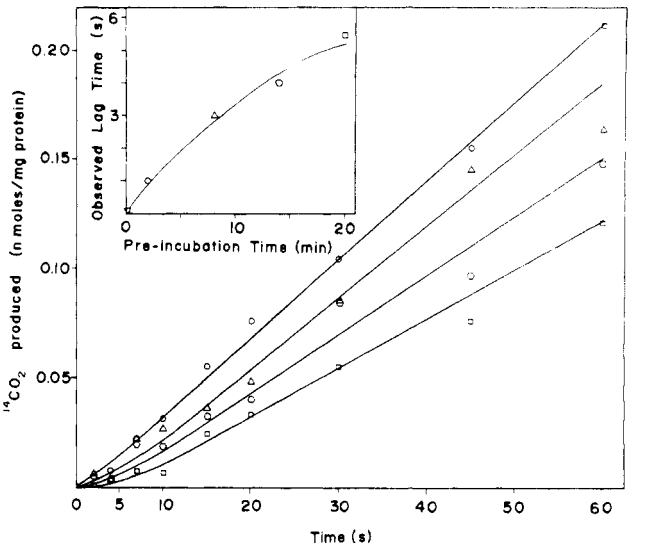

FIGURE 2: Initial velocity of OMP decarboxylase as a function of preincubation time when UMP synthase is preincubated in buffer. Assays were performed at 0 °C with UMP synthase at 3.6 mg of protein/mL, as described under Experimental Procedures. UMP synthase was preincubated in standard Tris–dithiothreitol buffer for 2 (O), 8 (Δ), 14 (○), or 20 min (□) before the OMP decarboxylase activity was initiated by the addition of [7-^{14}C]OMP. Extrapolation of the linear portion of each progress curve to the abscissa determines the observed lag time; these are replotted in the inset. UMP synthase in buffer alone exists as a 3.6S monomer.

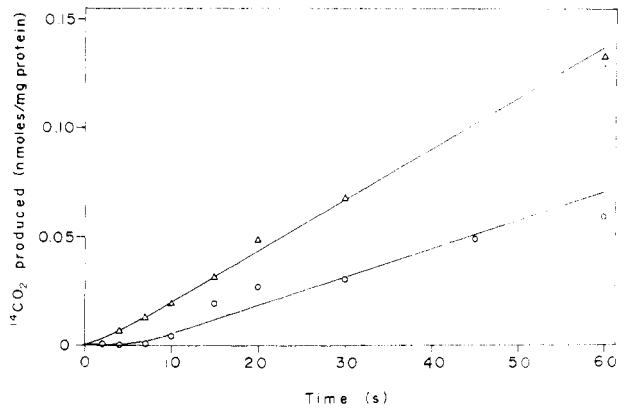

FIGURE 3: Initial velocity of OMP decarboxylase as a function of enzyme concentration when UMP synthase is preincubated in buffer. Assays were performed as described in the legend to Figure 2 with a preincubation time of 8 min and UMP synthase at final concentrations (mg of protein/mL) of 1.5 (O) and 4.5 (Δ).

If azaUMP (6-azaUMP) (0.1 μM) is included during the 20-min preincubation, it will convert UMP synthase predominantly to the 5.1S dimer. The OMP decarboxylase progress curve gives results intermediate between those observed with the 3.6S form (Figure 3) and the 5.6S form (Figure 4): with the 5.1S dimer, there is a clear lag in the appearance of enzyme activity similar to studies with the 3.6S form (Figures 2 and 3), but this lag is not related to enzyme concentration (Figure 5).

Since the experiment of Figure 5 included both OMP and azaUMP in the final reaction mixture, additional control experiments were done, repeating the conditions described for Figures 3 and 4, but starting the enzyme assay by the combined addition of [7-^{14}C]OMP and azaUMP (0.1 μM final concentration). The progress curves observed were not significantly different from those shown in Figures 3 and 4. This means that the results shown in Figure 4, where two effectors are present, can be directly compared with the results shown

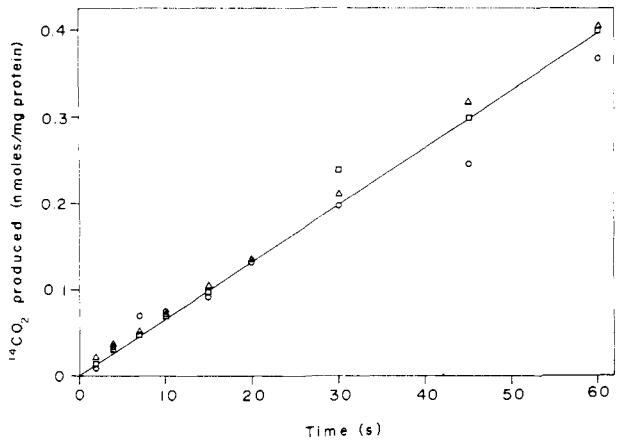

FIGURE 4: Initial velocity of OMP decarboxylase when UMP synthase has been preincubated with 1.0 μM OMP. Enzyme activity was assayed at 0 °C with UMP synthase at final concentrations (mg of protein/mL) of 1.5 (○), 3.0 (△), or 4.5 (□). UMP synthase was preincubated for 20 min in standard buffer and then for an additional 30 s with unlabeled OMP at 1.0 μM, after which enzyme activity is measured by the addition of [7-^{14}C]OMP (25 μM final concentration). UMP synthase in the presence of OMP is converted to the 5.6S dimer.

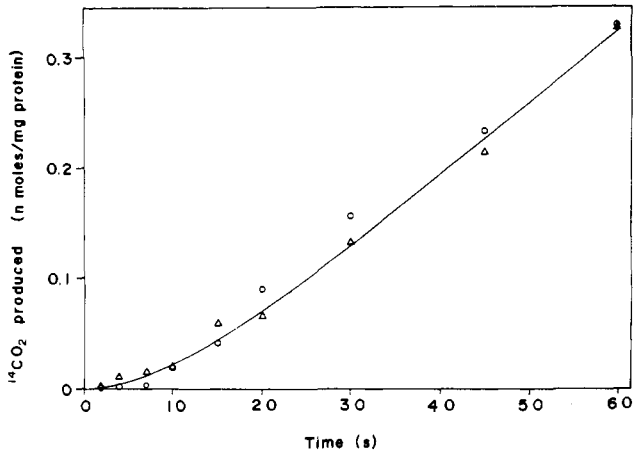

FIGURE 5: Initial velocity of OMP decarboxylase when UMP synthase has been preincubated with 0.1 μM azaUMP. Enzyme activity was assayed at 0 °C with UMP synthase at final concentrations (mg of protein/mL) at 1.5 (○) or 3.0 (△). Preincubation of UMP synthase in standard buffer plus 0.1 μM azaUMP was for 20 min. Under these conditions, UMP synthase exists predominantly as the 5.1S dimer.

in Figures 3 and 4, where only OMP is present.

If similar experiments are done while measuring the orotate phosphoribosyltransferase activity, there is no observable lag time is the progress curve, either as a function of the preincubation time (data not shown) or as a function of the concentration or the aggregation/conformation state of UMP synthase (Figure 6). UMP synthase was initially present completely as the 3.6S monomer (Figure 6A), or mostly as the 5.1S dimer (Figure 6B), or as the 5.6S dimer (Figure 6C). In all cases, there is no lag in the production of OMP from orotate. In each case, there is a lag in the appearance of UMP. This lag is observed since there is no OMP present at the start of the reaction, and the lag time for UMP formation represents the time required for orotate to be converted to OMP, which must then reach both the catalytic site of OMP decarboxylase and the regulatory site of UMP synthase whereby the 5.6S species, with active decarboxylase activity, is produced.

OMP Decarboxylase and Cooperativity. Many investigators have reported that OMP decarboxylase shows negative cooperativity at higher substrate concentrations, with a break in the double-reciprocal plot of V^{-1} vs. $[S]^{-1}$ at OMP con-

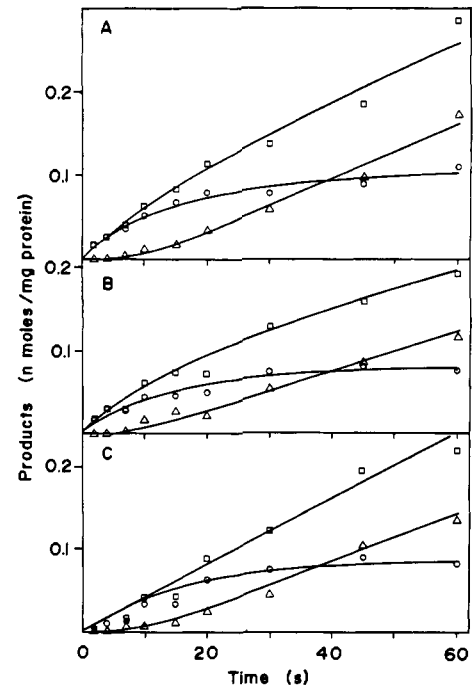

FIGURE 6: Initial velocity of orotate phosphoribosyltransferase when UMP synthase has been preincubated. Enzyme activity was assayed at 0 °C with UMP synthase (at a final concentration of 3.0 mg of protein/mL) preincubated for 20 min as described in the legends to Figures 2–5 in (A) standard buffer, (B) 300 μM P-Rib-PP, or (C) 1.0 μM OMP. These preincubation conditions produce, respectively, the 3.6S monomer, the 5.1S dimer, and the 5.6S dimer of UMP synthase. Products measured were (○) OMP, (△) UMP, and (□) sum of OMP + UMP.

centrations greater than about 10 μM. This has been observed with the enzyme from yeast and rat liver (Fyfe et al., 1973) and from human fibroblasts (Worthy et al., 1975). In addition, studies with human erythrocyte UMP synthase have reported two breaks in the curve (i.e., that the enzyme activity has three sets of V_{max} and K_m), with the second break in the curve occurring at OMP concentrations greater than 2 μM (Brown et al., 1975) or greater than 30 μM (Tax & Veerkamp, 1979).

In previous work with the enzyme from mouse Ehrlich ascites cells, such kinetics were never observed. Since dithiothreitol is an effective stabilizer of the orotate phosphoribosyltransferase activity, we have routinely included it during purification or assay of UMP synthase. Since the earlier studies in other laboratories focused mainly or exclusively on the OMP decarboxylase activity, dithiothreitol was not added to their enzyme preparations. We thought that this might be significant for the kinetic studies since dithiothreitol favors the formation of the 3.6S monomer with the Ehrlich ascites UMP synthase, while enzyme prepared without sulfhydryl reagents exists simultaneously as a stable mixture of monomer and dimers (Traut & Jones, 1979).

However, whether enzyme is purified and assayed with, or without, dithiothreitol, the K_m and V_{max} values observed are the same, and over the range of OMP concentrations of 5 nM to 5 μM the velocity and Michaelis constants of the OMP decarboxylase activity remain unchanged. Continued measurements at high OMP concentrations (5 μM to 5 mM) have not given consistent evidence of a break in the curve, although we have occasionally observed a break occurring when OMP concentrations were greater than 600 μM (data not shown).

Differential Stability of the Catalytic Domains of UMP Synthase. Since a variety of effector molecules has been shown to promote the formation of the 5.1S dimer or the 5.6S dimer from the 3.6S monomer, and since the data in Figures 2–5

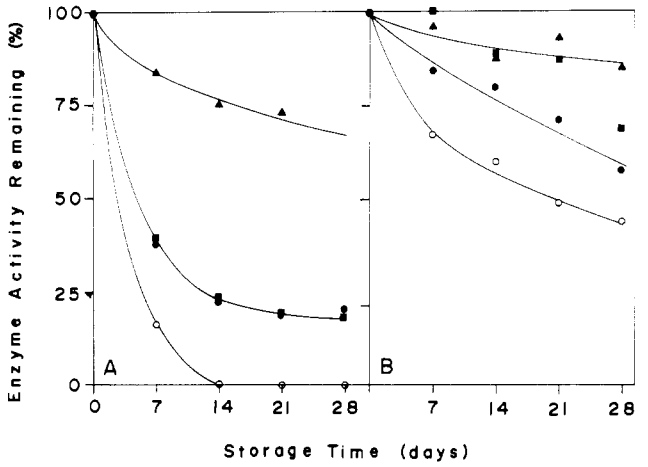

FIGURE 7: Long-term storage stability of UMP synthase activities at 4 °C. UMP synthase at a final concentration of 6.0 mg of protein/mL was stored in Tris–dithiothreitol buffer containing the following effectors: (●) none, (▲) 300 mM P_i, (■) 1 μM azaUMP, or only in Tris buffer (○). (A) Orotate phosphoribosyl transferase activity; (B) OMP decarboxylase activity.

suggest that the different aggregation/conformation species of UMP synthase have varied OMP decarboxylase activity, we tested the possibility that the different physical forms of UMP synthase would vary in their susceptibility to degradation or denaturation on storage. Enzyme was stored at 4 °C at a final concentration of 6 mg of protein/mL in buffer, with or without additional effectors. As shown in Figure 7, in the absence of dithiothreitol, the phosphoribosyltransferase activity was completely lost after 14 days, while 60% of the OMP decarboxylase activity remained. Addition of dithiothreitol (final concentration at 2 mM) decreased the loss of phosphoribosyltransferase activity, with an equivalent stabilizing effect on the decarboxylase activity. Clearly, if UMP synthase is stored at 4 °C in the monomer form, the phosphoribosyltransferase activity becomes quite labile.

Considerable protection is offered by ligands that bind to the catalytic sites. Inorganic phosphate (P_i) is a competitive inhibitor of both catalytic sites of UMP synthase[3] (Traut et al., 1980). At a concentration of 300 mM, P_i gives significant protection against loss of activity for both enzyme centers of UMP synthase. Since this concentration of P_i maintains UMP synthase principally in the dimer form (Traut & Jones, 1979), it would appear that the monomer is unstable while the dimer is relatively very stable for long-term storage. However, azaUMP offers no protection to the phosphoribosyltransferase activity; its rate of loss is the same as in buffer alone (Figure 7), while the OMP decarboxylase activity receives essentially similar stability from azaUMP and P_i. The significant difference between these two ligands is that azaUMP, at the concentration used, binds effectively only to the OMP decarboxylase site. Therefore, stability of the catalytic activities on long-term storage is not dependent on the physical aggregation state of UMP synthase but on the presence of a ligand that can bind to, and thereby stabilize, the catalytic site.

Discussion

Measurements of the two enzyme activities of UMP synthase show that only the OMP decarboxylase activity is linear over the range 37–0 °C (Figure 1A). Quite a range of interpretations have been proposed (Dixon & Webb, 1964) for a break in an Arrhenius plot, such as we observed for the phosphoribosyltransferase activity (Figure 1B). The interpretation that best fits our data is that the phosphoribosyltransferase catalytic domain exists in different conformations at temperatures above and below 7 °C. Such a conformational shift, with a concomitant break in the Arrhenius plot, has been demonstrated with histidine ammonia-lyase from *Pseudomonas* (McClard & Kolenbrander, 1974). We had previously shown that preincubation of UMP synthase with OMP at 37 °C resulted in an enhanced rate (about 2-fold) for the phosphoribosyltransferase activity (Traut & Jones, 1977). The absence of a similar effect with UMP synthase preincubated with OMP and at 0 °C (Figure 6C) may well be related to a temperature-sensitive conformational change as suggested by the break in the Arrhenius plot. Thus, our earlier studies suggest that the 5.6S dimer of UMP synthase has greater phosphoribosyltransferase activity at 37 °C while at 0 °C all forms of UMP synthase appear to have equivalent phosphoribosyltransferase activity.

The lag times observed for the OMP decarboxylase activity, when UMP synthase was preincubated in buffer as the 3.6S monomer, were dependent on the length of the preincubation period (Figure 2) and on the enzyme concentration (Figure 3). We were not able to measure lag times over an extensive range of enzyme concentrations because at UMP synthase concentrations greater than about 4 mg of protein/mL the lag time approaches zero, while at protein concentrations below 1 mg/mL there was insufficient product formed to give reliable values.

The experiments measuring OMP decarboxylase activity when UMP synthase was preincubated under conditions where it exists as either the 3.6S monomer (Figure 3) or the 5.1S dimer (Figure 5) suggest that the OMP decarboxylase activity of UMP synthase is significantly active only when UMP synthase is in the 5.6S form. Extrapolation of the linear portions of the progress curves to the abscissa gives intercept values that define the time, in seconds, for full OMP decarboxylase activity to appear. Since the rate of formation of the dimer is assumed to be dependent on the concentration of available enzyme, the following observations support the interpretation that only the dimer of UMP synthase, and specifically the 5.6S dimer, has significant OMP decarboxylase activity: the rate of dimerization would be decreased at lower enzyme concentrations and should therefore produce longer lag times, and this is exactly what we observed; the appearance of OMP decarboxylase activity should not be related to enzyme concentration when UMP synthase is preincubated in the 5.1S or 5.6S dimer forms, and this is exactly what we observed. Although we observed a lag time with the 5.1S form, the lag time is independent of enzyme concentration (Figure 5).

It is interesting that the lag time for the appearance of OMP decarboxylase activity was greater when UMP synthase was in the 5.1S dimer form than when it was preincubated as the 3.6S monomer. This suggests that the 5.6S dimer is more easily formed from the 3.6S monomer than from the 5.1S dimer. Since the transition from the 3.6S monomer to the 5.6S dimer involves one or more conformational changes in addition to a dimerization step, the above observation suggests that the conformational change that distinguishes the 5.6S dimer from the 5.1S dimer may be achieved more rapidly with the monomer. That is, the sequence conformational change of monomer followed by dimerization → 5.6S is more rapid than the sequence dimerization of monomer → 5.1S followed by conformational change → 5.6S.

[3] The competitive inhibition by P_i of both the orotate phosphoribosyltransferase and the OMP decarboxylase activities was first observed by Dr. P. R. Kavipurapu.

The appearance of the phosphoribosyltransferase activity was instantaneous under all conditions and did not vary with enzyme concentration or the physical state of UMP synthase imposed by the preincubation conditions (Figure 6). The appearance of UMP under these conditions always shows a lag, but this is the time required for a minimal concentration of OMP to be produced, and to initiate conversion of the 3.6S monomer to the active 5.6S dimer in some cases. This time has been referred to as the transient time (cf. Welch & Gaertner, 1975). The first detectable UMP synthesis occurred after 4 s, when OMP concentrations had reached about 0.075 μM, and maximum UMP synthesis normally occurred by 10 s, when the average OMP concentration had become 0.15 μM.

Under conditions where the synthesis of UMP is dependent on the availability of OMP synthesized by the phosphoribosyltransferase activity of UMP synthase, one would expect the transient time to decrease at higher enzyme concentrations, since the required concentration of OMP should be attained more quickly. This has been demonstrated for the *arom* multienzyme system (Welch & Gaertner, 1975) and was generally observed in our studies. Under conditions similar to those in the experiment of Figure 6B, the transient time was 11 s when UMP synthase was at 1.5 mg of protein/mL and 9 s with UMP synthase at 3.0 mg of protein/mL. Under the conditions depicted in Figure 6C, transient times were 16, 12.5, and 10 s at respective enzyme concentrations of 1.5, 3.0, and 4.5 mg of protein/mL. However, under the conditions of Figure 6A, transient times were fairly constant at 10 s for different concentrations of UMP synthase.

The transient times observed with UMP synthase, when measured at 0 °C, were an average of about 10 s. We were unable to detect a definite transient time at 37 °C, where our resolution is about 1 s; this suggests that in vivo the transient time would be approximately $\leq$ 1 s. This very rapid transient time for orotate $\rightarrow$ OMP $\rightarrow$ UMP is in good agreement with earlier observations on the ability of UMP synthase to channel efficiently the intermediate, OMP, in the synthesis of UMP from orotate (Traut & Jones, 1977; Traut, 1980).

The experiments where we measured OMP decarboxylase activity as a function of substrate concentration also support our interpretation that the 5.6S dimer is the principal species of UMP synthase that is active in decarboxylating OMP. By measuring enzyme velocity at OMP concentrations as far below K_m as possible, we thought that the enzyme might not be completely converted to the 5.6S species and that the velocity measured might reflect the activity of a mixed population of monomers and dimers, or even of monomers alone since the lowest concentration of OMP required for the 3.6S $\rightarrow$ 5.6S transition has not been definitively established. Were this premise true, however, we should have seen a break in the V^{-1} vs. $[OMP]^{-1}$ plot at OMP concentrations near K_m (0.23 μM) or higher since OMP concentrations of this magnitude are adequate for maintaining essentially all of UMP synthase in the 5.6S form (Traut & Jones, 1979). We have found, however, that there is no change in the V_{max} or K_m of the OMP decarboxylase activity over a range of OMP concentrations from 5 nM to 500 μM.

Since our preincubation studies show a lag time for the appearance of OMP decarboxylase activity (Figures 2 and 3), suggesting a differential activity of the dimer relative to the monomer, a break in the velocity curve in the range of OMP concentrations that are known to promote the transition of monomer to dimer would have helped to establish if the monomer of UMP synthase had any decarboxylase activity. Currently, we have no direct evidence that the monomer does have decarboxylase activity. However, we cannot definitely exclude the possibility that the monomer has some decarboxylase activity. It may be that the anticipated break in the velocity curve would occur in the pM range of OMP concentrations, but we currently cannot measure that low.

We have occasionally observed a break in the velocity curve at very high OMP concentrations (>600 μM); this has not been a consistent observation and varies with different preparations of UMP synthase. In any case, negative cooperativity at such high OMP concentrations is not related to a monomer–dimer transition. Since our enzyme preparation is far from pure, this may reflect a secondary decarboxylase activity due to a contaminating protein. Alternatively, this may imply negative cooperativity for an enzyme where normally only half of the sites are active, as discussed by Levitzki & Koshland (1976). We do not have adequate data to distinguish between these cases at present. Studies with UMP synthase from different tissues have also reported multiple V_{max} and K_m values for OMP decarboxylase as a function of OMP concentration. Generally, breaks in the velocity curve occurred at OMP concentrations far lower than we have observed, being in the range 2–30 μM with UMP synthase from rat liver (Fyfe et al., 1973), human fibroblasts (Worthy et al., 1975), or human erythrocytes (Brown et al., 1975; Tax & Veerkamp, 1979). In fact, the studies with human erythrocytes have each reported three separate K_m values for different ranges of OMP concentration, and Brown et al. (1975) have attributed their values as being respectively associated with a monomer, dimer, and tetramer of UMP synthase. Again, all of the above studies were done with impure enzyme preparations, and there are not enough data to give a definitive interpretation for these observations.

One of the functions that the regulation of a monomer–dimer system can produce is altered enzymatic activity, as has been discussed above. A second possibility is that the dimer has enhanced stability against degradation. As measured by long-term storage stability at 4 °C, maintenance of UMP synthase in the dimer form (by the addition of ligands) does not, by itself, result in the stabilization of both catalytic sites against loss of activity (Figure 7). Rather, it appears that stabilization only occurs when a specific ligand is bound to a catalytic site and that the two sites of UMP synthase are independent in this respect. However, most of the ligands thus far examined interact with both catalytic sites of UMP synthase.

In conclusion, it has previously been shown that UMP synthase may exist in at least three different physical states, a 3.6S monomer, a 5.1S dimer, and a 5.6S dimer, and that various ligands, but principally OMP, are effectors that provide the conversion of monomer to the 5.1S or 5.6S dimer. Regulation of these monomer–dimer transitions may be physiologically significant since our current studies suggest that OMP decarboxylase activity is either uniquely, or predominantly, associated with the 5.6S dimer of UMP synthase while previous studies suggest that the phosphoribosyltransferase activity is enhanced in the 5.6S dimer (Traut & Jones, 1977). Of physiological compounds, OMP is the most important as a promotor of the active 5.6S species. Inorganic phosphate is, so far, the only compound that blocks this activating effect of OMP.

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pH Kinetic Studies of Bovine Brain Hexokinase†

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ABSTRACT: The variation of kinetic parameters with pH was examined for bovine brain hexokinase with glucose and MgATP as substrates. The $-\log V_1$ and $-\log (V_1/K_m)$ profiles for both substrates were examined and seen to decrease below pH 6.5. All profiles asymptotically approached slopes of −1, indicating that the loss of activity in each instance was due to the protonation of a single group on the enzyme. Analysis of the data indicated two ionizable groups were involved in the reaction. One functions in the binding of ATP and in catalysis while the other participates in the binding of glucose. The $-\log V_1$ profiles both showed a "hump" attributed to a loss of activity in the pH region 7.5–5.5. Addition of aluminum ions to the reaction mixture increased the magnitude of the hump, but the inhibition was abolished by the addition of citrate. Kinetic studies carried out at pH 7 indicated that aluminum was a competitive inhibitor with respect to ATP and noncompetitive with respect to glucose. However, secondary plots of the kinetic data were nonlinear, concave downward, indicating that the inhibition is not of a simple type. Possible explanations for this phenomenon are presented.

Brain hexokinase (hexokinase I) is now recognized as a primary control point of glycolysis in the brain. Because of its metabolic importance, much time and study have been devoted in attempts to acquire a thorough understanding of the kinetic mechanism of hexokinase (Purich et al., 1973). However, it has only been within the last few years that attention has been directed to the chemistry of this enzyme's active site. Recent studies have shown that sulfhydryl groups are required for activity in both yeast (Jones et al., 1975; Otieno et al., 1975) and mammalian (Redkar & Kenkare, 1972; Chou & Wilson, 1974; Subbarao & Kenkare, 1977a) hexokinase, and there is some evidence to indicate that these groups are involved in the binding of substrate (Subbarao & Kenkare, 1977b). Carboxyl groups have been reported to be involved in the action of yeast hexokinase (Pho et al., 1974), and from X-ray crystallographic data Anderson et al. (1978) have observed what seems to be an interaction between an aspartyl residue and the 4- and 6-hydroxyl groups of the sugar substrate. There is evidence for the involvement of histidyl residues in the active site of the wheat germ enzyme (Higgins & Easterby, 1974), but this residue has been found not to be involved in either the binding of substrate or the catalysis of yeast hexokinase (Grouselle et al., 1973).

In this study, we have made use of pH kinetics to gain information about the groups responsible for binding and catalysis of substrate molecules by bovine brain hexokinase. We report evidence for a single ionizable group involved in the catalysis of the reaction and in the binding of ATP and a second residue that functions in the binding of the carbohydrate substrate. In addition, we offer observations on an anomalous loss of activity below pH 8 similar to the decrease reported in a recent paper by Viola & Cleland (1978) on the pH profile of yeast hexokinase. Colowick has suggested that this inhibition may be caused by the trivalent metal ion Al^{3+}.[1] Our results indicate that this ion may indeed be responsible for the pH-dependent activity loss. Kinetic evidence indicates that this inhibition is nonlinear competitive with ATP and may be due to the formation of a strongly inhibitory aluminum–ATP complex, as well as interactions of the metal with the enzyme alone.

Experimental Procedures

Materials. Glucose-6-phosphate dehydrogenase was purchased from Boehringer Mannheim. Buffers used in the experiments were from Calbiochem. ATP and $NADP^+$ were products of Sigma, and glucose was from Pfhanstiehl. Distilled deionized water was used in the preparation of all reagents. All other chemicals were of the highest purity available commercially.

Methods. Bovine brain hexokinase was prepared by the method of Redkar & Kenkare (1972) and had a specific ac-

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[1] Dr. S. P. Colowick, personal communication.

0006-2960/80/0419-6074$01.00/0