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ATP:UMP–CMP PHOSPHOTRANSFERASE FROM *TETRAHYMENA PYRIFORMIS*

II. KINETIC STUDIES AND REACTION MECHANISM WITH UMP*

THOMAS Q. GARVEY, III**, F. K. MILLAR AND E. P. ANDERSON

Laboratory of Biochemistry and Laboratory of Physiology, National Cancer Institute, National Institutes of Health, Bethesda, Md 20014 (U.S.A.)

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SUMMARY

Partially purified UMP kinase (ATP:UMP phosphotransferase, EC 2.7.4.4) from *Tetrahymena pyriformis* has been investigated with regard to reaction mechanism. From data on isotope exchange in the partial reactions, it was concluded that the reaction proceeds by a sequential mechanism and that a ternary complex of the enzyme with both substrates is formed as the central intermediate in the reaction. Initial velocity studies, varying both UMP and ATP concentrations, gave a complex pattern, primarily complicated by the fact that the reaction was inhibited except at very low levels of ATP·Mg. This inhibition was apparently predominantly competitive with UMP.

INTRODUCTION

UMP kinase (ATP:UMP phosphotransferase, EC 2.7.4.4) catalyzes the reaction

$$UMP + ATP \rightleftharpoons UDP + ADP \quad (1)$$

The enzyme was partially purified from *Tetrahymena pyriformis*, a flagellated protozoan that does not synthesize pyrimidines or purines *de novo*, and is, therefore, dependent on synthesis of nucleotides from preformed pyrimidine and purine rings. Preliminary studies in our laboratory on the characteristics of the UMP kinase reaction were undertaken and reported[2]. Further investigation on the mechanism of the UMP kinase reaction was stimulated by the knowledge that nucleoside diphosphatekinase (EC 2.7.4.6), catalyzing phosphate transfer between nucleotides by the type reaction, XDP + YTP ⇌ XTP + YDP, purified from either human erythrocytes[3–5] or yeast[6], has been shown to behave by a ping-pong mechanism of substrate

* Portions of this work have been presented in a preliminary report[1].

** Present address: Department of Medicine, Georgetown University Horpital, Washington, D. C., U.S.A.

addition and product release. On the other hand, the mechanism of adenylate kinase (EC 2.7.4.3) purified from yeast (catalyzing the myokinase reaction, AMP + ATP $\rightleftharpoons$ 2 ADP) appears to be a random sequential addition of the two substrates to the enzyme to form a ternary complex[7]. It was, therefore, thought to be of interest to determine the mechanism for UMP kinase, another nucleotide phosphorylation enzyme.

In bisubstrate reactions, equilibrium isotope exchange in the half-reactions can distinguish between a ping-pong and a sequential mechanism[8,9]. The studies undertaken were aimed at discovering whether or not UMP kinase catalyzed the two half-reactions (2) and (3) outlined below*:

$$\begin{aligned} &\text{ATP} + E \rightleftharpoons \text{ADP} + E\text{–}P \\ &[^{14}\text{C}]\text{ADP} + E\text{–}P \rightleftharpoons [^{14}\text{C}]\text{ATP} + E \\ \text{Sum:}\ &\text{ATP} + [^{14}\text{C}]\text{ADP} \rightleftharpoons \text{ADP} + [^{14}\text{C}]\text{ATP} \end{aligned} \qquad (2)$$

and

$$\begin{aligned} &\text{UDP} + E \rightleftharpoons \text{UMP} + E\text{–}P \\ &[^{14}\text{C}]\text{UMP} + E\text{–}P \rightleftharpoons [^{14}\text{C}]\text{UDP} + E \\ \text{Sum:}\ &\text{UDP} + [^{14}\text{C}]\text{UMP} \rightleftharpoons \text{UMP} + [^{14}\text{C}]\text{UDP} \end{aligned} \qquad (3)$$

For a sequential mechanism in which formation of a ternary complex is obligatory, neither half-reaction would be catalyzed by the enzyme. Conversely, if UMP kinase proceeds by a ping-pong mechanism, then both half-reactions should be observed, proceeding independently and at rates comparable to that of the full reaction. Thus, incubations of the half-reactions, employing a sensitive assay for the theoretical products, should yield evidence for or against a ping-pong mechanism[8,9]. The evidence presented in this paper indicates that the UMP kinase reaction proceeds by a sequential rather than a ping-pong mechanism.

MATERIALS AND METHODS

Non-radioactive nucleotides were obtained as the sodium salts from Sigma Chemical Co., Calbiochem, P–L Laboratories, or Boehringer Mannheim Corp.; they were used without further purification. [2-^{14}C]UMP (spec. act. 0.05 Ci/mmole) and [^{3}H]ATP (spec. act. 5.64 Ci/mmole) were purchased from International Chemical and Nuclear Corp. [8-^{14}C]ATP (spec. act. 0.02 Ci/mmole) was purchased from New England Nuclear. [8-^{14}C]ADP (spec. act. 0.039 Ci/mmole) was a product of Schwarz/Mann. Sephadex G-75 was obtained from Pharmacia. Thin-layer sheets of polyethyleneimine cellulose on plastic film were purchased from J. T. Baker Chemical Co. Oxoid yeast extract came from Consolidated Laboratories, Inc., Chicago Heights, Ill., and Proteose Peptone No. 3 from Difco Laboratories, Detroit, Mich. Bovine serum albumin was the product of Armour Laboratories, Chicago, Ill. Liquifluor and Triton X-100 were from New England Nuclear.

Enzyme source

Cultures of *Tetrahymena pyriformis*, strain W, were graciously supplied by Dr

* *E*, enzyme; *E–P*, phosphorylated enzyme.

George Kidder and Dr Virginia Dewey. Cultures were maintained and grown for harvesting in a medium containing 2% Proteose Peptone, 1% dextrose, and 0.5% Oxoid yeast extract. In a typical purification, a loop (about 0.05 ml) of stationary phase culture was transferred to 1 l of fresh medium kept in a 4-l Fernbach flask. Four such inoculations were made for the usual harvest, and the organism was then grown axenically in the dark at 25–28 °C and harvested on the fifth day while still in late log phase growth.

UMP kinase was purified by methods previously developed in this laboratory[2]. The final preparation off Sephadex G-75 SF was 300-fold purified over the starting material, a crude lysate*. It phosphorylated 15–25 μmoles UMP/min per mg protein and had a protein concentration of 0.18–0.27 mg/ml. This preparation was used for kinetic studies of the enzyme.

Enzyme assay

UMP kinase activity was routinely assayed as conversion of radioactive UMP to UDP**. The standard incubation mixtures contained [2-^{14}C]UMP, ATP, $MgCi_2$ and enzyme protein as indicated, in 0.03–0.05 M potassium phosphate buffer at pH 7.4, which is the pH optimum of the enzyme[2]. These assay conditions were based on the known properties of the enzyme, which appears to be stabilized by phosphate[2] and requires a divalent cation. Experiments indicated that either Mg^{2+} or Mn^{2+} satisfied this requirement; Mn^{2+} was apparently as effective as Mg^{2+}. For some experiments enzyme activity was alternatively assayed as conversion of radioactive ATP to ADP. In these cases the incubation mixture contained, instead, [8-^{14}C]ATP and unlabeled UMP *plus* the other components. In experiments in which it was necessary to compare conversions of UMP and ATP, [2-^{14}C]UMP and [^{3}H]ATP were both included in the incubations.

Aliquots of the incubation mixture were removed at specific time intervals, and the reaction was terminated by pipetting these aliquots into tubes (preheated in a boiling water bath) which were then kept in boiling water for 1 min. After this, the sample tubes were placed in ice or frozen until the sample was chromatographed. An aliquot was then pipetted onto a thin-layer sheet of polyethyleneimine cellulose. The polyethyleneimine cellulose was stored in the dark at −18 °C and prior to use any degraded polyethyleneimine was removed by washing the sheets twice by ascending chromatography in distilled water, followed by air drying. The substrate and product nucleotides were then separated by ascending chromatography in 1.0 M LiCl for 2.5 h. The nucleotide spots on the chromatogram were localized either by radioautography or by ultraviolet fluorescence (Mineralight). In later experiments, radioautography was not performed routinely since excellent agreement between the two methods was demonstrated. In order to localize with the Mineralight, 10–20 nmoles of each relevant nucleotide standard were applied to each sample channel on

* Unpublished studies have shown that it is possible to purify UMP kinase activity further from the Sephadex G-75 SF fraction using hydroxyapatite columns. This procedure gave about 2-fold purification over that already achieved. These studies were not, however, carried beyond a preliminary exploration.

** Or to UDP *plus* UTP for less pure enzyme fractions. The final fraction off Sephadex usually showed no detectable conversion of UDP to UTP in the routine 10-min assay. The final purified fraction also contained no adenosine triphosphatase activity in the absence of other added nucleotides.

the thin-layer polyethyleneimine cellulose sheets and co-chromatographed with the sample in each case. Following ultraviolet localization, nucleotide spots were cut from the sheet and immersed directly in 20 ml of scintillation counting fluid (Liquifluor, 4 g PPO and 50 mg POPOP per l toluene). In order to minimize the effects of size of the fragment and its orientation in the counter, the chromatographic technique was adjusted to produce nucleotide localization in areas small enough to cover only the bottom of the counting vial (a circular piece of about 1.5-cm diameter). Samples were counted in a Nuclear Chicago Mark I scintillation counter using the channels ratio method to correct for quenching; the variation in counting efficiency was only about 2%.

This chromatographic method was satisfactory whenever only one labeled compound was present in the incubation mixture, *i.e.* it produced adequate separation of the mono-, di-, and triphosphates of any one nucleoside. However, when [^{3}H]ATP as well as a ^{14}C-labeled uridine nucleotide was present in the incubation mixture, the sample was chromatographed in duplicate, once with the set of uridine nucleotide standards, and once with the adenosine nucleotide standards. In addition, better resolution was required and was attained by using serial ascents (without drying between changes of liquid phase) in 0.5 M, 1.0 M, and 1.5 M LiCl, of 5, 50 and 40 min, respectively. Following this serial chromatography, the thin-layer sheets were air dried and then heated for 10 min at 60 °C. Nucleotide areas were then localized as before. The spots were deliberately kept small and the cut out areas were placed in the bottom of counting vials, covered with 2 ml of 0.5 M HCl, shaken, allowed to stand for 30 min and then covered with 20 ml of Triton–toluene (1:2, v/v), containing 57 ml Liquifluor in 1500 ml. After addition of the counting fluid, the vials were shaken until clearing occurred, and the samples were counted as described above. Counting efficiency was uniform and the variation was about 1–2%.

The percent conversion of UMP to UDP was calculated from the radioassay, and enzyme activity was expressed as μmoles pyrimidine nucleoside monophosphate phosphorylated per min/mg protein. Appropriate corrections were made in the calculations for contamination of the labeled substrate, as supplied from the distributor, with non-substrate radioactivity; these corrections were based on chromatography of the starting substrate at the time of each experiment.

An enzyme unit was defined as that quantity of protein that would convert 1 μmole of UMP to UDP in 1 min. Protein concentration in the enzyme preparations was estimated by the method of Lowry *et al.*[10], with bovine serum albumin as the standard.

Data processing

Data derived from the radioactivity assays were punched on paper tape and processed with an IBM-1620 computer for evaluation of the initial velocity studies. The double reciprocal plots of $1/v$ against $1/[S]$ were fitted by the least-squares method.

RESULTS

Isotope exchange in the partial reactions

In 5-min assays of the UMP kinase reaction, ADP production was usually

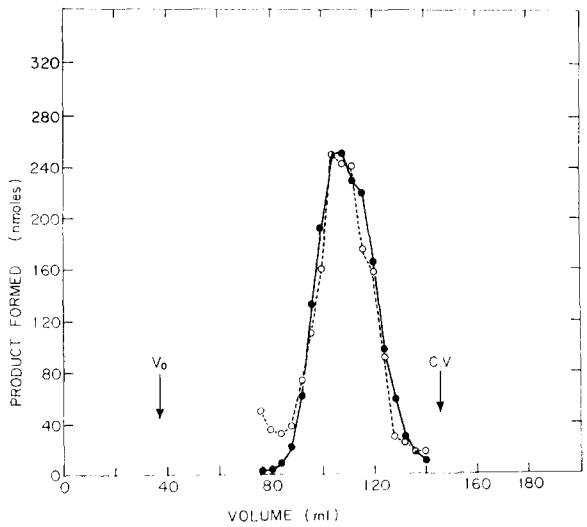

Fig. 1. UDP production and ADP production by UMP kinase eluted from Sephadex G-75 SF. The column was eluted with 0.1 M potassium phosphate buffer (pH 7.4). The column volume (C.V.) was 145 ml, the void volume (V_0) was 37 ml, and the flow rate was 4 ml/h. Each tube for determination of UMP phosphorylation (●—●) contained, in a volume of 50 μl, 2.5 μmoles potassium phosphate buffer (pH 7.4), 150 nCi[2-^{14}C]UMP, 0.5 μmole unlabeled UMP, 0.5 μmole unlabeled ATP, 0.6 μmole $MgCl_2$, and 25 μl of the column effluent. Each tube for determination of ADP production (○---○) contained the same except that [2-^{14}C]UMP was omitted and 50 nCi [^{3}H]ATP were included. These data are based on 5-min assays at 28 °C, and the figures are expressed as nmoles product formed per 50 μl/5 min.

nearly stoichiometric with UMP phosphorylation. These data are shown in Fig. 1, which illustrates the elution of UMP kinase from Sephadex G-75 SF, as assayed (5 min) by both UDP and ADP production. Although some extra production of ADP was evident in fractions just ahead of the UMP kinase peak, the fractions within the peak of kinase activity showed nearly stoichiometric formation of UDP and ADP.

To determine whether or not the UMP kinase reaction proceeds by a ping-pong or a sequential mechanism, isotope exchange studies in the half-reactions were undertaken using equilibrium concentrations of each substrate–product pair. As a preliminary step, it was established that UMP kinase activity persisted to at least 6 h at 28 °C. An estimate of the equilibrium concentrations of the four reactants in the full reaction (ATP, ADP, UMP and UDP) was then obtained from 5.5-h incubations starting with initial concentrations of 10 mM UMP and 5 mM ATP (Fig. 2). Although ATP formation from ADP may have been slightly greater than UMP phosphorylation at the longer time intervals, product formation was again nearly stoichiometric. The incubation mixture, when sampled at 15 min, 30 min, 1 h, 2 h and 5.5 h, approached an extrapolated equilibrium in which nearly 400 nmoles of each product was formed (2 mM). This represented equilibrium concentrations of 2.0 mM UDP, 8.0 mM UMP, 2.0 mM ADP, and 3.0 mM ATP. These concentrations were therefore used for isotope exchange studies on each of the two half-reactions, *i.e.* [^{14}C]ADP *plus* unlabeled ATP (Reaction 2, above) and [^{14}C]UMP *plus* unlabeled UDP (Reaction 3).

The results indicated that although near stoichiometry had been observed in the full reaction (Figs 1 and 2), differences did appear in sensitive assays of the half

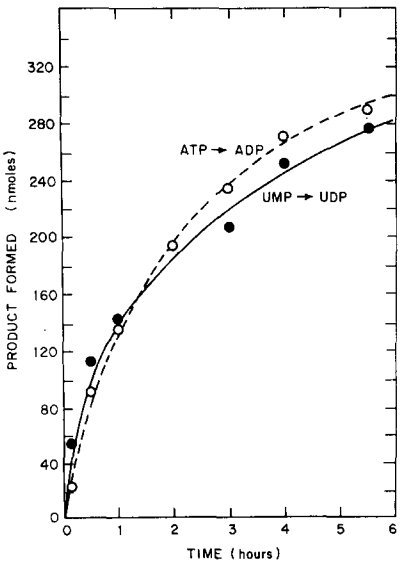

Fig. 2. Estimation of equilibrium for the UMP kinase reaction. Each tube contained, in a volume of 200 μl, 10 μmoles potassium phosphate buffer (pH 7.4), 100 nCi [2-^{14}C]UMP, 80 nCi [^{3}H]ATP, 2 μmoles unlabeled UMP, 1 μmole unlabeled ATP, 1.2 μmole $MgCl_2$, and 6 μg enzyme protein. The reaction was run at 28 °C over a period of 5.5 h. Both half-reactions were assayed as described under Methods. [^{14}C]UMP phosphorylation to [^{14}C]UDP (●—●); [^{3}H]ATP conversion to [^{3}H]-ADP(○---○). Each point represents the mean of three values derived from separate assays. The coefficients of variation for these points ranged from 3.3 to 24.2% with the higher values evident in the estimation of ADP formation at longer time intervals.

reactions. However, neither of the half-reactions, in the absence of the other substrate, proceeded at an appreciable rate in comparison with the full reaction. Starting with 8 mM [^{14}C]UMP and 2 mM unlabeled UDP, there was no detectable incorporation of label into UDP during a 15-min incubation period in four separate experiments. After 5.5-h incubation, this was still true. With initial concentrations of 2 mM [^{14}C]-ADP and 3 mM unlabeled ATP, based on four separate experiments, the incorporation of labeled ADP into ATP over a 15-min period was 55 $\pm$ 19 (mean $\pm$ S.D.) nmoles incorporated per min/mg protein. During an equivalent incubation period, the full reaction, starting with 10 mM [^{14}C]UMP and 5 mM ATP, proceeded at a rate of 605 $\pm$ 78 (mean $\pm$ S.D.) nmoles UMP converted to UDP per min/mg protein. When allowed to proceed for periods up to 5.5 h, the incorporation of [^{14}C]ADP into ATP in the half reaction continued at a uniform slow rate. In contrast, the full reaction with [^{14}C]UMP and ATP proceeded at the much more rapid rate up to about 12% conversion and more slowly thereafter. In many experiments with various preparations of the enzyme, the exchange of label between [^{14}C]UMP and UDP (Reaction 3) was always too low to be detected, and that between [^{14}C]ADP and ATP (Reaction 2) proceeded at a rate that was 2–10% of the rate of the full reaction (Reaction 1).

A mechanism for a reversible reaction in which only one half-reaction occurs, and not the other, has not been observed in experiments with pure enzyme preparations (Cleland, W. W., personal communication). Furthermore, since the rate of half-reaction 2 was much less than that of the full reaction, and since this conversion could also be catalyzed by nucleoside diphosphatekinase, the likelihood was that

UMP kinase was catalyzing neither half-reaction 2 nor 3 and that the "exchange" observed in Reaction 2 was actually a true phosphate transfer reaction between ATP and [^{14}C]ADP. To test the hypothesis of a nucleoside diphosphate kinase contaminant, further studies were conducted to see if the two activities could be dissociated. Eluant from the Sephadex G-75 column was collected in 1-ml aliquots and assayed in four different ways. UMP kinase was assayed in the usual way as conversion of [^{14}C]UMP to [^{14}C]UDP in the presence of ATP. The presence of adenosine triphosphatase activity was tested by measuring the dephosphorylation of [^{3}H]ATP to [^{3}H]ADP in the absence of any other added nucleotide. Nucleoside diphosphate kinase activity was assayed as conversion of [^{14}C]ADP to [^{14}C]ATP with GTP as phosphate donor. In addition, conversion of [^{14}C]ADP to [^{14}C]ATP was measured in the presence of unlabeled ATP, as in the assay for half-reaction 2. This conversion could, of course, also have been catalyzed by nucleoside diphosphate kinase.

The results of these experiments are presented in Fig. 3 and show that the two activities could be dissociated. A distinct peak of UMP kinase activity could be seen at 92–102 ml of effluent, while ahead of this appeared a peak of nucleoside diphosphate kinase activity, as assayed either by transphosphorylation between GTP and [^{14}C]ADP or as conversion of [^{14}C]ADP to [^{14}C]ATP. It was concluded that the so-called "exchange" between [^{14}C]ADP and unlabeled ATP, half-reaction 2, observed in the UMP kinase preparation, could almost surely be attributed to contaminating nucleoside diphosphate kinase trailing into the UMP kinase fraction from the

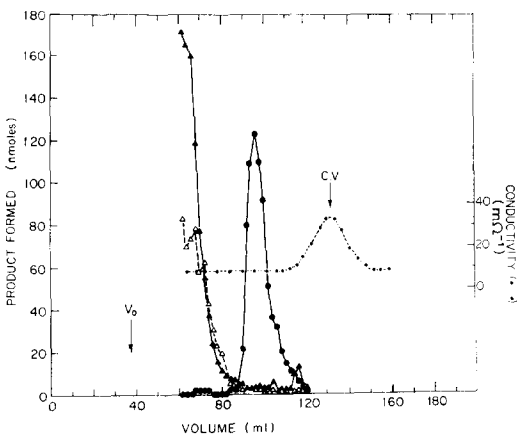

Fig. 3. Elution from Sephadex G-75 SF of UMP kinase and of contaminating nucleoside diphosphate kinase. The column was eluted with 0.1 M potassium phosphate buffer (pH 7.4). The column volume (C.V.) was 133 ml, as indicated by the conductivity pattern (●---●), the void volume (V_0) was 37 ml, and the flow rate was 4 ml/h. Assays were begun when the effluent volume was 62 ml. Each tube for assay of UMP kinase (●—●) contained, in a volume of 75 μl, 3 μmoles potassium phosphate buffer (pH 7.4), 376 nCi [2-^{14}C]UMP, 0.75 μmole unlabeled UMP, 0.38 μmole ATP, 0.82 μmole $MgCl_2$, and 15 μl of the column effluent. For the assay of nucleoside diphosphate kinase as transphosphorylation between [^{14}C]ADP and GTP (▲—▲), each tube contained, in a volume of 75 μl, 3 μmoles potassium phosphate buffer (pH 7.4), 188 nCi [^{14}C]ADP, 0.38 μmole unlabeled ADP, 0.75 μmole GTP, 0.82 μmole $MgCl_2$, and 15 μl of the effluent. For assay of transphosphorylation between [^{14}C]ADP and ATP (△---△), each tube contained, in 75 μl, 3 μmoles potassium phosphate buffer (pH 7.4), 188 nCi [8-^{14}C]ADP, 0.15 μmole unlabeled ADP, 0.23 μmole ATP, 0.82 μmole $MgCl_2$, and 15 μl of the column effluent. These results were all based on 10-min assays at 28 °C, and the results are expressed as nmoles product formed per 75 μl/10 min. UMP kinase activity was pooled from the 92–102 ml fractions.

earlier diphosphate kinase peak (*cf.* also Fig. 1). As previously noted, dephosphorylation of [^{3}H]ATP was found to be absent in the UMP kinase peak; it was also absent in all but the earliest fractions assayed in the nucleoside diphosphate kinase peak (not shown in Fig. 3).

Linearity of the reaction with protein concentration

Initial velocity studies were undertaken to confirm that the UMP kinase reaction proceeded by a sequential mechanism[8,9,11]. Fig. 4 shows the initial velocity (1-min) of the UMP kinase reaction over a range of enzyme concentrations for two different levels of the substrates ([UMP] = [ATP] = 1 and 10 mM). The data show that the velocity was proportional to the quantity of enzyme added over a protein concentration range from 0.003 to 0.05 mg/ml at both levels of substrate, and over a broader range at the 1 mM substrate level. However, early time points were found to be crucial, since the velocity curves plateaued within 1 to 2 min at most protein concentrations with the low level of substrates. Moreover, at low levels of enzyme, the conversion was very low throughout the time course of the reaction, and not enough product was formed to be reliably measured. Thus, relatively high enzyme levels within the linear range were always used for the kinetic studies. Also, reaction

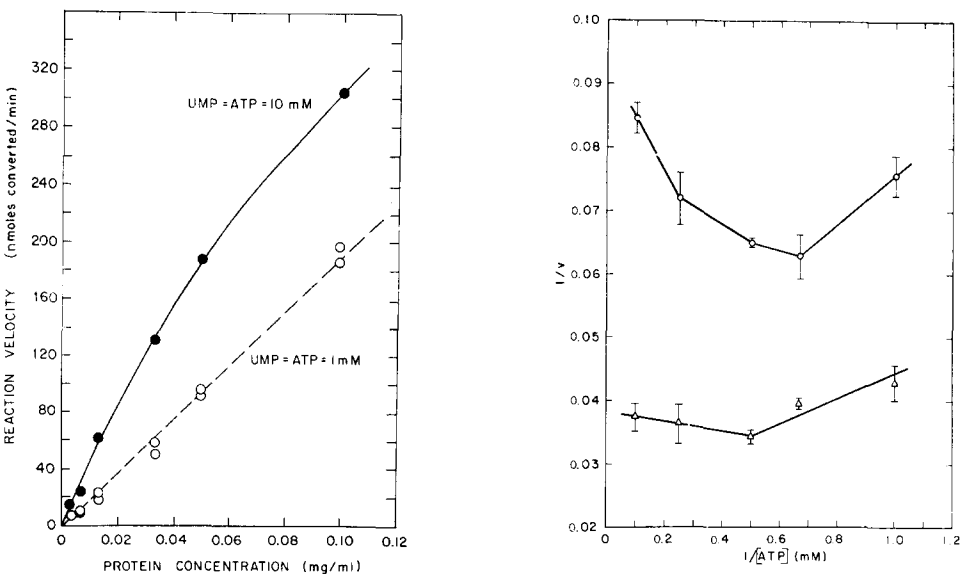

Fig. 4. Initial UMP kinase reaction velocity as a function of protein concentration at two different levels of substrate. The reaction mixtures contained, in a volume of 100 μl, 3 μmoles potassium phosphate buffer (pH 7.4), 100 nCi [2-^{14}C]UMP, either 0.1 (○---○), or 1.0 (●—●) μmole unlabeled UMP and ATP as indicated, and $MgCl_2$ at a level 0.1 μmole in excess of the starting level of ATP. Initial velocity was considered to be that during the first minute of a 5-min incubation at 28 °C. Velocities are expressed as total nmoles UMP converted per min in the 100-μl reaction mixture. Conversion was measured as described under *Enzyme assay*.

Fig. 5. Double reciprocal plot of initial velocity (μmoles UMP phosphorylated per min/mg protein), *vs* ATP concentration (mM) at 2 levels of UMP (○—○, 1 mM UMP; △—△, 4 mM UMP). $MgCl_2$ was 1 mM excess over the starting concentration of ATP. Protein concentration was 0.3 μg/100 μl of incubation mixture. Reaction was run at 28 °C. Initial velocities are based on the first-minute conversion, assayed in triplicate.

within the first minute, or a maximum of 10% conversion of substrate to product, was considered to be the limit within which an experimentally derived velocity could be considered "initial".

Initial velocity pattern

Initial velocity studies were carried out varying each substrate over a concentration range from 1.0 mM to 10.0 mM. Mg^{2+} was added to 1.0 mM excess over the initial ATP concentration, as recommended by Cleland[8] for kinetic studies, and standard assay conditions were used.

The initial velocity pattern was primarily informative with regard to inhibition at high substrate levels. Our earlier observation[2] that the UMP kinase reaction is inhibited in the presence of high levels of ATP·Mg was explored further. Fig. 5 shows a double reciprocal plot (1/initial reaction velocity against 1/ATP concentration) for 1 mM and 4 mM UMP. Inhibition of the reaction is evident at the two highest levels of ATP, but it is also apparent that this inhibition was affected by the

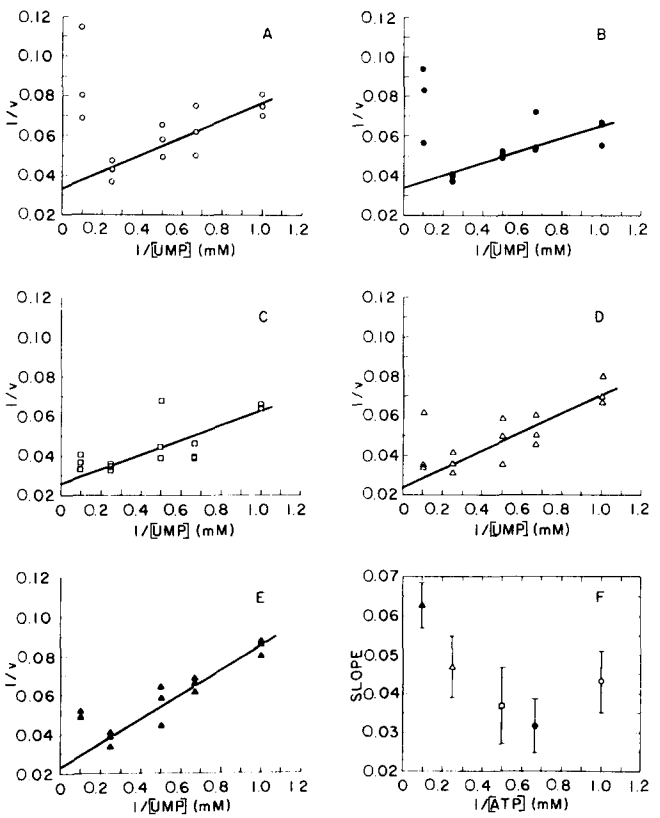

Fig. 6. A–E. Double reciprocal plots of initial velocity (μmoles UMP phosphorylated per min/mg protein) *vs* UMP concentration (mM). (A) ○—○, 1 mM ATP; (B) ●—●, 1.5 mM ATP; (C) □—□, 2 mM ATP; (D) △—△, 4 mM ATP; (E) ▲—▲, 10 mM ATP. In each case the UMP concentration range was 1–10 mM. The experiment was the same as that used for Fig. 5. (F) Replot of slopes of Figs 6A–E plotted against the reciprocal of the ATP concentration.

UMP concentration. Thus, inhibition by these levels of ATP was greater at 1 mM UMP than at 4 mM. Also, the ATP level that produced optimum reaction velocity was higher with 4 mM UMP than with 1 mM UMP.

The full data from this experiment have also been plotted in Fig. 6, with UMP as the varied substrate and ATP as the changed fixed substrate. The resulting double reciprocal plots all show evidence of UMP inhibition also, at the highest (10 mM) level of UMP ($1/v$ *vs* $1/[UMP]$, Figs 6A–6E), and in each case these points have been omitted from the least-squares fit of the line for $1/v$ *vs* $1/[UMP]$ to the data. The calculated slopes of these lines are plotted against the reciprocal of the changed fixed substrate in Fig. 6F. Again this shows the inhibition at higher levels of ATP. However, at lower levels of ATP (between 1.0 and 1.5 mM), there is evidence of a positive correlation between the reciprocal of the fixed substrate concentration and the slope of the line of $1/v$ *vs* $1/[UMP]$. Additional initial velocity experiments were carried out at lower substrate levels down to 0.1 mM, and the pattern was qualitatively the same. These results constitute further evidence for a sequential and not a ping-pong mechanism in the reaction for UMP phosphorylation.

DISCUSSION

This study was undertaken to determine whether the UMP kinase reaction proceeds by a sequential or a ping-pong mechanism. To date most kinases that have been investigated kinetically have been found to catalyze reactions characterized by a sequential mechanism of substrate addition[8]. These include creatine kinase (EC 2.7.3.2) from either rabbit muscle[12,13] or calf brain[14], arginine kinase (EC 2.7.3.3) from crayfish muscle[15], yeast[16] and mammalian[17,18] hexokinases (EC 2.7.1.1), *Escherichia coli* galactokinase (EC 2.7.1.6) (ref. 19), pyruvate kinase (EC 2.7.1.40) from rabbit muscle[20], and yeast adenylate kinase[7]. The conclusions with regard to reaction mechanism have been based on initial velocity patterns and on the results of product inhibition studies and equilibrium isotope exchange. In most cases the mechanism appears to be one of random sequential addition, *i.e.* not requiring an obligatory order of substrate addition.

In contrast, based on results obtained with similar methods, it has been concluded that the reactions catalyzed by *E. coli* acetate kinase (EC 2.7.2.1) (refs 21 and 22), by phosphoglycerate kinase (EC 2.7.2.3) from rabbit muscle[23], and by nucleoside diphosphate kinase from either mammalian erythrocytes[3–5] or yeast[6], proceed by a ping-pong mechanism of substrate addition and product release. In these reactions a phosphoenzyme intermediate has been indirectly demonstrated, or isolated, and shown to donate phosphate to a substrate of the reaction to form a product.

UMP kinase is an ATP-dependent nucleoside phosphate kinase, like both adenylate kinase and nucleoside diphosphate kinase. Data from the present studies indicate that UMP kinase acts by a sequential mechanism, like adenylate kinase, rather than by a ping-pong mechanism, like nucleoside diphosphate kinase. This conclusion is drawn primarily from the results of isotope exchange in the partial reactions. Such studies can, in a straightforward fashion, provide convincing evidence for or against a ping-pong reaction mechanism[8,9]. The UMP kinase reaction is readily reversible, and proceeds in the reverse direction at a rate not very different from that

of the forward reaction. Thus, whereas ATP acts as a phosphate donor in the forward reaction, UDP contributes a phosphate to ADP in the reverse reaction. If the UMP kinase reaction is then postulated to proceed by a ping-pong mechanism, it follows that the enzyme should catalyze phosphate transfer between the members of each isolated substrate–product pair, *i.e.* UMP–UDP and ADP–ATP. A necessary condition for the conclusion that the mechanism is ping-pong would be the observation of both half-reactions, each proceeding at a rate equivalent to that of the full reaction. In these studies the UMP kinase reaction was begun with the substrate and product at predetermined, putative equilibrium concentrations, and no chemical reaction due to the kinase should have been occurring. Since we were unable to demonstrate UDP–UMP phosphate transfer catalyzed by UMP kinase in the absence of the adenosine nucleotide, the finding of slow "exchange" between ADP and ATP was apparently the result of a contaminating activity. The subsequent demonstration of a broad peak of nucleoside diphosphate kinase activity eluted off Sephadex just in front of the UMP kinase peak with demonstrable trailing, indicated that the UMP kinase off Sephadex was indeed contaminated with quantities of nucleoside diphosphate kinase sufficient to produce the observed ATP–ADP phosphate transfer. The final conclusion from these studies was that UMP kinase catalyzed phosphate exchange in neither of the half-reactions, and that, therefore, the full reaction proceeds by a sequential rather than a ping-pong mechanism.

The initial velocity pattern confirms this conclusion, but it alone would not have established the reaction mechanism. This was partly because of error in the method, and partly because of the inhibition of the reaction at high substrate levels. Errors were undoubtedly introduced by the time-point assay technique; continuous monitoring of reaction rate is always preferable for kinetic studies[24]. However, a continuous assay, employing an indicator system of phosphoenol pyruvate, pyruvate kinase, lactate dehydrogenase (EC 1.1.1.27), and NADH, coupled to ADP production in the UMP kinase reaction, was investigated and had to be discarded because UDP was a substrate for pyruvate kinase[25,26], and the affinity of this enzyme for UDP was sufficiently different from that for ADP to produce a complicated kind of change in the absorbance as the UMP kinase reaction proceeded*. Nevertheless, with the radioactivity time-point assay, at non-inhibitory levels of substrates, the slopes for $1/v$ against $1/[S]$ increased with the reciprocal of the fixed substrate concentration (Figs 5 and 6). This was also true at substrate levels below those shown. These results are consistent with a sequential mechanism for the reaction, in agreement with the data from the isotope exchange in the partial reactions.

The initial velocity studies showed that the reaction was inhibited at high levels of either substrate. At the higher levels of ATP·Mg there was clear-cut inhibition that was not independent of the concentration of the UMP substrate. It is not

* The V for UDP phosphorylation by pyruvate kinase was observed by us to be about 30% that of the V with ADP as substrate. Moreover, the relative contributions of the two diphosphates to the change in absorbance varied with their concentrations in a complicated fashion. Thus, the change in absorbance at 340 nm produced by coupling the enzyme systems was found to be a relatively complex function of the changing concentrations of both diphosphate products of the UMP kinase reaction. This difficulty was not observed with CMP as substrate for the kinase. CDP was converted to CTP by pyruvate kinase with a V only 1–2% that of the ADP to ATP conversion. Thus, this coupled system can be successfully employed in studying the kinetics of the nucleoside monophosphate kinase with CMP as phosphate acceptor (Garvey, III, T. Q. and Anderson, E. P., unpublished observations).

yet clear whether this represents substrate inhibition, product inhibition by the diphosphates formed, or inhibition by some competing ionic species of ATP that is not a substrate of the enzyme. This enzyme has been found to be sensitive to product inhibition[2], and this might serve as a regulatory mechanism[27].

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