Reversible ATP-dependent Transition between Two Forms of Human Cytosolic Thymidine Kinase with Different Enzymatic Properties

(Received for publication, October 14, 1992, and in revised form, February 23, 1993)

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Human cytosolic thymidine kinase, subunit molecular mass about 24 kDa, is a tetramer in the presence of ATP but a dimer in the presence of thymidine or without substrates. The pure, substrate-free enzyme showed complex, non-hyperbolic thymidine substrate kinetics with an apparent $K_m$ of 15 μM. Incubation with ATP at 4 °C induced a time-dependent transition to an enzyme form with hyperbolic kinetics and a 20-fold lower $K_m$ value for thymidine (0.7 μM) but the same maximal velocity as for cytosolic thymidine kinase (TK1) without ATP. Removal of the ATP by carboxymethyl chromatography reestablished the non-hyperbolic kinetics with the low affinity for thymidine ($K_m$ instead = 12 μM), and this enzyme form could be reversed once more by ATP incubation to the high affinity enzyme form. Similar shifts could not be induced by thymidine. The activating effect of ATP depended on the concentration of enzyme protein in a linear manner. These results indicate that ATP is a positive effector of cytosolic thymidine kinase, controlling a kinetically slow transition between two molecular forms of the enzyme. A hypothetical reaction mechanism is presented to explain the complex kinetic behavior.

The cytosolic thymidine kinase, TK1 (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.21) is a cell cycle-regulated enzyme in the nucleoside salvage pathway rescuing thymidine from extra- and intracellular catabolic processes. Despite a considerable number of investigations, the exact role of the enzyme has not yet been clarified. A close correlation between the thymidine kinase activity and the proliferative state of the cell has been established, and the fluctuation of thymidine kinase activity during the cell cycle is more pronounced than of other enzymes associated with the DNA synthesis (Kitt, 1976; Munch-Petersen and Tyrsted, 1977; Piper et al., 1980; Sherley and Kelly, 1988a). Several investigations have shown that the changes in TK1 mRNA during the cell cycle are too limited to account for the pronounced fluctuations in enzyme activity (Coppock and Pardee, 1987; Sherley and Kelly, 1988b; Ito and Conrad, 1990). This indicates that translational and posttranslational modifications are predominant in regulating thymidine kinase activity in cycling cells. Recently, it has been shown that amino acid residues near the C-terminal end are responsible for degradation of thymidine kinase protein in the G1 and M phase, and that mutations in this part of the gene allow expression in G0 cells (Kauffman and Kelly, 1991; Kauffman et al., 1991).

During the years, there have been many reports on the properties of human TK1, with diverging results and observations. Native molecular weights between 45,000 and 200,000 have been reported for the enzyme from leukemic cells (Lee and Cheng, 1976a; Sherley and Kelly, 1988a; Munch-Petersen, 1990), human placenta (Ellims et al., 1982; Gan et al., 1983; Tamiya et al., 1989), and lymphocytes (Munch-Petersen, 1984; Munch-Petersen et al., 1991). There have been indications that ATP induces polymerization of the enzyme (Munch-Petersen, 1984; Tamiya et al., 1989; Munch-Petersen et al., 1991), and cooperative and non-cooperative substrate and inhibition kinetics have been reported (Lee and Cheng, 1976b; Gan et al., 1983; Munch-Petersen, 1984; Munch-Petersen et al., 1991). Recently, TK1 was purified to homogeneity from HeLa cells (Sherley and Kelly, 1988a) and from human lymphocytes (Munch-Petersen et al., 1991), and in both reports, the native enzyme in the presence of ATP was a tetramer of 24-kDa polypeptide. To clarify the previously reported divergences, we decided to study the kinetics on the pure thymidine kinase after removal of substrates and discovered that the enzymatic properties of the substrate-free enzyme differed markedly from previous observations. Furthermore, we found that incubation of the substrate-free enzyme with ATP induced a slow transition to a more active kinase with more than 20-fold higher substrate affinity. Removal and readdition of ATP showed that the effect of ATP on the kinetic behavior and the substrate affinity was completely reversible.

EXPERIMENTAL PROCEDURES

Chemicals—CHAPS was purchased from Boehringer Mannheim, CM-Sepharose fast flow from Pharmacia, and unlabeled nucleosides and nucleotides from Serva and Sigma. All other reagents were commercial preparations of the highest purity available. [methyl-3H]Thymidine (925 GBq/mmol) was obtained from the Radiochemical...
where and linear for at least 60 min when less than 10% of the initial substrate concentration.

that can phosphorylate 1 pmol of nucleoside per min at 37 °C under

sayed by the DEAE-cellulose paper method as previously described (Munch-Petersen et al., 1984). Standard assay conditions were: 50 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 10 mM dithiothreitol, 50 mM mercaptoethanol, and 200 μM thymidine. Buffer D: 10 mM Tris-HCl (pH 7.6), 10% glycerol, 5 mM MgCl₂, 5 mM dithiothreitol. Buffer E: 50 mM Tris-HCl (pH 8.0), 10% glycerol, 5 mM MgCl₂, 5 mM dithiothreitol, 0.5 mM CHAPS, and 0.1 M KCl. Buffer F: 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 5 mM mercaptoethanol, and 0.1 M KCl.

Thymidine Kinase Assay—The thymidine kinase activity was assayed by the DEAE-cellulose 81 paper method as previously described (Munch-Petersen, 1984). Standard assay conditions were: 50 mM Tris-HCl, pH 8.0 (22 °C), 2.5 mM MgCl₂, 10 mM dithiothreitol, 50 mM mercaptoethanol, 2.5 μM radiolabeled thymidine, 10% glycerol, 5 mM MgCl₂, 5 mM dithiothreitol, and 0.5 mM CHAPS, and 0.1 M KCl. The thymidine kinase activity in the fractions was determined as described under “Experimental Procedures.” Before each experiment, the column was rinsed with more than 10 column volumes of buffer F. The molecular mass markers (+) are from left to right: alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 12.4 kDa. Inset, relation between M₀ and Kₐᵥ = (vₒ - vₐ)/(vₒ - vₐ) for the five marker proteins (C); Kₐᵥ for TK1-ATP and TK1+ATP (O). vₒ, vₐ, v₀, and vₐ are the elution volume, the void volume, and the bed volume, respectively.

Substrate Kinetics—For characterizing and comparing the specificity of TK1 toward different substrates and inhibitors, and for clarifying the molecular mechanism, it is necessary to perform the experiments on substrate-free enzyme. Previous attempts to remove thymidine from purified TK1 by gel filtration resulted in great loss of enzyme activity probably due to dilution of the enzyme protein. In the present work, we used CM-Sepharose chromatography by which thymidine was removed and the enzyme concentrated in a single step, improving the yield considerably. Furthermore, immediate addition of ATP to a final concentration of 2.5 mM to an aliquot of the eluted TK1 increased the yield more than 2-fold. Hereafter, the TK1 stored in ATP is designated TK1+ATP, and the substrate-free TK1 is designated TK1-ATP.

For further studying the effect of ATP on the enzyme, we incubated substrate-free TK1 at a concentration of 200 ng/ml, with 2.5 mM ATP with or without 6 μM thymidine at 4 °C for various time intervals, and assayed the enzyme activity at 37 °C. A typical result of such an experiment is shown in Fig. 2 and demonstrates a more than 3-fold increase in thymidine kinase activity after 2 h of incubation. Furthermore, the presence of thymidine seems to increase the rate of activation. On the other hand, no activation was obtained when incubating the enzyme with thymidine alone or without substrates. The activation was strongly dependent in a linear manner on the concentration of enzyme protein (Fig. 2, inset), and no activation was obtained below 10 ng/ml. This implies that no

**FIG. 1. The effect of ATP on the apparent native molecular weight of TK1.** 0.3 ng of pure, substrate-free TK1 (TK1-ATP) was injected on the Superose 12 column preequilibrated with buffer F (○). 0.3 ng of TK1-ATP was preincubated for 30 min with 2 mM ATP prior to injection on the Superose 12 column preequilibrated with buffer F containing 2 mM ATP (○). The thymidine kinase activity in the fractions was determined as described under “Experimental Procedures.” Before each experiment, the column was rinsed with more than 10 column volumes of buffer F. The molecular mass markers (+) are from left to right: alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 12.4 kDa. Inset, relation between M₀ and Kₐᵥ = (vₒ - vₐ)/(vₒ - vₐ) for the five marker proteins (C); Kₐᵥ for TK1-ATP and TK1+ATP (○). vₒ, vₐ, v₀, and vₐ are the elution volume, the void volume, and the bed volume, respectively.

**RESULTS**

**Molecular Weight Properties**—The effect of ATP on the native molecular weight of TK1 is demonstrated in Fig. 1. In the absence of ATP, the peak of activity eluted with an apparent molecular weight of 56,000. The presence of 2 mM ATP during chromatography increased the apparent molecular weight from 56,000 to 120,000. Since the subunit molecular mass of TK1 is 24-26 kDa, the native enzyme can exist as a dimer or tetramer. The elution profiles are not completely homogenous, and a shoulder at the front of the TK1 peak, as seen when eluted in the absence of ATP, and a tail seen when eluted in presence of ATP may reflect an equilibrium between a dimer and a tetramer form of the enzyme.

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**Molecular Weight Determination**—The apparent molecular weight of native TK1 was determined as previously described on a Superose 12 column (10 x 300 mm) connected to a fast protein liquid chromatography system (Pharmacia) (Munch-Petersen et al., 1991).
ATP Controlled Shifts between Two Forms of Thymidine Kinase 1

The effect of ATP on the activity of TK1. 20 ng of pure, substrate-free TK1 (TK1-ATP) was incubated at 4°C in 0.1 ml of buffer E containing 2.5 mM ATP (●) or 2.5 mM ATP plus 6 μM thymidine (○). At the indicated times, aliquots of the incubation mixture were diluted 50-fold in buffer E and assayed at 37°C for thymidine kinase activity with 1 μM thymidine at standard assay conditions. Inset, TK1-ATP were incubated 1 h at the indicated enzyme concentrations at 4°C in buffer E containing 2.5 mM ATP. The enzyme activity was assayed at 37°C by addition of 0.1 ng of the incubated enzyme to 50 μl of standard reaction mixture.

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Fig. 3. The relation between rate of dTMP formation and concentration of TK1-ATP at different thymidine concentrations. The initial velocities at the indicated amounts of TK1-ATP per 50 μl of assay mixture were measured at 0.1 μM (●), 1 μM (■), and 10 μM (▲) thymidine.

Incubation of TK1+ATP for 1 h at 4°C reversed the biphasic kinetics of TK1-ATP to rectangular hyperbolic kinetics as indicated by the linear Hofstee plot. The initial velocities of the incubated enzyme measured at low thymidine concentrations were increased about 2-fold, whereas at saturating thymidine concentrations the initial velocities of TK1-ATP and the incubated TK1 were almost equal. Further storage of the incubated enzyme in the incubation mixture at -70°C for 2 weeks resulted in complete reversal to the kinetics obtained with TK1+ATP, since the straight line in the Hofstee plot superimposed the line obtained with TK1+ATP. From Hill plots of the data (Fig. 4, inset), S0.5 values of 0.7 and 15 μM were calculated for TK1+ATP and TK1-ATP, respectively, indicating that TK1+ATP has a more than 20-fold higher affinity for thymidine than TK1-ATP. The Hill coefficient for TK1-ATP was 0.74, indicating an apparent negative cooperative reaction mechanism, but increased to 1.0 after 1 h of incubation with ATP. The Hill coefficient for TK1+ATP was 1.17.

Incubation of TK1-ATP at 100 μM thymidine for 2 h at 4°C did not change the thymidine substrate kinetics as indicated by the biphasic Hofstee plot (Fig. 4). The Hill plot of these data superimposed the plot obtained with TK1-ATP, and S0.5 was 14 μM and the Hill coefficient was 0.74. The biphasic kinetic pattern was retained during further storage in thymidine.

We then examined the effect of removing ATP from TK1+ATP by a second chromatography on CM-Sepharose. An aliquot of the resulting, substrate-free TK1, designated TK1-ATP/2, was made 2.5 mM with ATP and designated TK1+ATP/2. Similar kinetic experiments as those depicted in Fig. 4 were performed with the rechromatographed enzyme forms, and the results are shown in Fig. 5. The Hofstee curves and the Hill plots clearly indicate that the effect of ATP on TK1 is reversible, since removal of ATP reestablished the biphasic pattern with the low thymidine affinity, and a second incubation with ATP reestablished the rectangular hyperbolic pattern with the high thymidine affinity. The S0.5 values and Hill coefficients were 12 μM and 0.73 for TK1-ATP/2, 1.4 mM and 0.96 for TK1-ATP incubated with ATP, and 1 μM and 1.0 for TK1+ATP/2, respectively.

The biphasic kinetics of TK1-ATP was retained at various assay concentrations of ATP between 0.025 and 5 mM, as indicated by the parallel lines in the Hill plots (Fig. 6) and Hill coefficients between 0.75 and 0.8 (Fig. 3). The S0.5 values increased from 14 to 130 μM thymidine, when the assay concentration of ATP decreased from 5 to 0.025 mM.

In a similar series of experiments with TK1+ATP (not shown), the S0.5 values at ATP concentrations between 5 and...
The Hill equation.

Simultaneous presence of ATP and thymidine (Sherley and Kelly, 1991) as well as in the simultaneous presence of ATP and thymidine (Sherley and Kelly, 1988a), indicating that the active enzyme is a tetramer. Thymidine alone is unable to induce tetramerization (Munch-Petersen, 1984). ATP is frequently applied in late purification steps because of the well-known stabilizing effect on the kinase activity. In the present work the concentration of enzyme obtained by using CM-Sepharose chromatography to remove substrates from purified TK1 made it possible to observe the ATP-activating effect, an effect that according to our results shown in Fig. 2 cannot be obtained with lower concentrations of enzyme protein. We were thus able to demonstrate that ATP is not only a stabilizer of thymidine kinase activity but a positive effector inducing a time-dependent increase in activity and a change in the kinetic reaction mechanism.

Furthermore, together the experiments in Figs. 2–5 indicate that the great loss of activity often seen in attempts to remove substrate from pure thymidine kinase may be the result of an enzyme concentration-dependent shift from a more active tetrameric form to a less active dimer, rather than a denaturation of enzyme protein.

The effect of ATP is reversible, since complete removal of ATP from TK1+ATP by a second chromatography on CM-Sepharose fully reestablished the biphasic kinetics with the low thymidine affinity (Fig. 5). Furthermore, reincubation with ATP once more reversed the biphasic kinetics to linear kinetics with the high thymidine affinity.

The ATP-activating effect is dependent on the enzyme concentration and should not occur at the applied standard assay concentrations of enzyme of 2 ng/ml (Fig. 2). However, the similar velocities obtained at saturating thymidine concentrations with the two enzyme forms, TK1-ATP and TK1+ATP, indicate that at high thymidine concentrations, a transition between the two enzyme forms takes place during the assay, although we always observe time-dependent linearity in the product formation. On the other hand, since it is technically impossible to follow product formation within the first minute of reaction and since we and other research groups always observe a “background radioactivity proportional to the amount of enzyme and radioactive substrate, it is possible that after mixing the assay components, a steady-state equilibrium is obtained within the first minute, with steady-state concentrations of complexes between substrates and dimer or tetramer enzyme forms depending on the initial substrate and enzyme concentrations.

The observed non-linear Hofstee plots and double-reciprocal plots may be explained by negative cooperative kinetics or by the occurrence in the reaction mixture of two enzymes with different substrate affinities, catalyzing the same reaction. Since ATP induces tetramerization of a dimer to a more active enzyme form, it is very likely that the non-linear kinetic plots are better explained assuming the presence of two enzymes.

We have tried to explain our observations in terms of a simplified kinetic reaction mechanism based on the following presumptions: 1) the reaction between ATP and thymidine can take place with a dimer as well as a tetramer form of thymidine kinase; 2) the tetramer has a higher catalytic activity than the dimer; 3) the rate of tetramerization is proportional to the concentration of complex between the dimer and ATP or both substrates; and 4) both enzyme forms combine with substrates to ternary complexes by a sequential ordered pathway with ATP as the first bound substrate. We suggest the following in vitro reaction mechanism. At low thymidine and saturating ATP concentrations, the steady-state amounts of complexes formed between dimer and one or both substrates are low and converted to free dimer and products, prior to the slower transition to the tetramer. The overall reaction will be dominated by the dimer with the lower

**DISCUSSION**

We have investigated the kinetic properties of pure human cytosolic thymidine kinase, TK1, and found that ATP, besides being a co-substrate, is a regulator of the enzymatic reaction mechanism and the level of catalytic activity in vitro. Previously, it has been shown that TK1 is a tetramer in the presence of ATP (Munch-Petersen, 1984; Sherley and Kelly, 1988a; Munch-Petersen et al., 1991) as well as in the simultaneous presence of ATP and thymidine (Sherley and Kelly, 1988a), indicating that the active enzyme is a tetramer.
catalytic rate. At increasing thymidine concentrations, the concentrations of the dimer-substrate(s) complex(es) increase, the tetramerization becomes significant, and the products will also be released from the tetramer. Once formed, the tetramer will be stabilized by ATP in the reaction mixture. Eventually, at saturating thymidine concentrations, almost all enzyme molecules will be on the tetramer form, and we will observe nearly the same velocity with TK1-ATP as with TK1+ATP, which was in the tetramer form prior to assay. The proposed reaction mechanism is further supported by the observations that the activating effect of ATP and the velocity at low thymidine concentrations are dependent on the enzyme concentration.

It is noticeable that ATP is also a positive effector of another cell cycle-regulated DNA precursor enzyme, the ribonucleotide reductase (Thelander and Reichard, 1979).

It can be speculated whether the reaction mechanism has any significance in vivo. The intracellular pool of ATP is in the range of 0.6–2 mM (Ferraro et al., 1992), and the intracellular thymidine concentration is nearly the same as the extracellular (Plageman and Wohlueter, 1980), in the range of 0.1–1.2 μM (Holden et al., 1980), but there are no reports about cell cycle-correlated fluctuations in these pools. However, the approximate level of TK1 protein can be estimated to about 4 μg/ml in phytoheamagglutinin-stimulated lymphocytes, as calculated from a TK activity of 55 nmol/min/10^6 cells (Munch-Petersen, 1990), a specific activity of 9500 nmol/min/mg (Munch-Petersen et al., 1991), 36% cells in S phase (Kofoed et al., 1986), and an average cell diameter of 20 μm. The overall activity is decreased about 15–400-fold (Munch-Petersen, 1990) to about 90 ng/ml in G1 cells and 30 ng/ml in G0 cells (cell diameter, 10 μm). The real figures are probably lower due to the presence of the mitochondrial thymidine kinase. According to Fig. 2, this indicates that the dimer form of TK1 is present in G1 and G0 cells. It is possible that the transition between the low affinity dimer and high affinity tetramer form of thymidine kinase is a fine tuning of the mechanism reported by Kauffman et al. (1991) to control the level of thymidine kinase protein in cycling and quiescent cells.

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