Modulation of the Feedback Regulation of Thymidine Kinase Activity by pH in 647V Cells

Miguel A. Vazquez-Padua, Keith Kunugi, Concepción Risueño, and Paul H. Fischer

Department of Human Oncology, University of Wisconsin Clinical Cancer Center, University of Wisconsin School of Medicine, Madison, Wisconsin 53792

ABSTRACT

We have studied the effect of pH on the interactions between thymidine kinase, thymidine triphosphate, and 5'-aminomido-2',5'-dideoxythymidine (5'-AdThd) in purified preparations of the enzyme and in intact 647V cells, a human bladder cancer cell line. Thymidylate kinase is competitively inhibited by 5'-AdThd. dTTP feedback inhibits in a noncompetitive fashion. However, 5'-AdThd partially reverses the inhibition produced by dTTP resulting in enhanced enzyme activity. We have found that dTTP (pKₐ = 7.5) is a much more potent inhibitor of purified preparations of thymidine kinase activity at low pH conditions. For example, 2.5 μM dTTP inhibited thymidine kinase activity by 50, 85, and 95% at pH values of 8.0, 7.5, and 6.5, respectively. The interaction of 5'-AdThd (pKₐ = 8.5) at either the active (competitive) or the regulatory (deinhibition) site is not altered significantly over a pH range of 6.5 to 9.5. To extend these findings to intact cells, we studied the perturbation of the uptake of thymidine by 5'-AdThd in 647V cells incubated in media buffered at various pH values. In cells exposed to media buffered at pH 8.5 or 7.5, 5'-AdThd maximally stimulated thymidine uptake about 250 and 300% at 10 and 30 μM, respectively. However, at pH 6.5, 300 μM 5'-AdThd was required to produce maximal stimulation of about 500%. These observations are consistent with the greater sensitivity of thymidine kinase (in situ) to feedback inhibition by dTTP at the lower pH conditions. Intracellular dTTP pool sizes were not affected by variations in pH during the short time course of our experiments. However, after 1 h, the intracellular concentration of 5'-AdThd was twice that of the extracellular medium in conditions at pH 7.5 and 8.5 but was equimolar across the membrane at pH 6.5. This does not account for the differences in the perturbation of thymidine uptake by 5'-AdThd at various pH values. In general, our results indicate that regulation of thymidine kinase by dTTP is pH dependent, while its modulation by 5'-AdThd is not, and that regulation of thymidine kinase in situ is sensitive to alterations in pH.

INTRODUCTION

Thymidine kinase catalyzes the first step in the salvage pathway of thymidine. Its activity is regulated by the feedback inhibition exerted by the end-product of the pathway, dTTP (2-4). This regulatory mechanism can limit the phosphorylation of cytotoxic nucleoside analogues substrates of thymidine kinase such as iododeoxyuridine (5), fluorodeoxyuridine (6), and trifluorothymidine (7). In fact, we have recently shown that feedback inhibition of thymidine kinase is an important factor in the limitation of the activation of fluorodeoxyuridine in the human bladder cancer cell line 647V (8).

We are interested in evaluating the feasibility of antagonizing this feedback regulatory mechanism to enhance the uptake, cytotoxicity, and antiviral activity of nucleoside analogues (9, 10). 5'-Aminothymidine can accomplish this by a direct interaction with the enzyme (11, 12). Using purified preparations of thymidine kinase from 647V cells we have found that this interaction is critically dependent on the presence of the feedback inhibitor, dTTP. In its presence, 5'-AdThd can enhance thymidine kinase activity by reducing the inhibition exerted by dTTP at the regulatory site. When present at high concentrations or in the absence of dTTP, 5'-AdThd inhibits thymidine kinase activity by competitive interactions at the active site. 5'-AdThd is not phosphorylated by the mammalian thymidine kinase (13) and it is not cytotoxic (14).

In elucidating the mechanism of action of 5'-AdThd, it is important to discriminate between interactions occurring at the active versus the regulatory site of the enzyme. Prusoff and Chang (15) reported that thymidine kinase derived from Ehrlich ascites carcinoma cells is regulated by dTTP in a pH-dependent manner, the enzyme activity being more inhibited at lower pH values. Both dTTP and 5'-AdThd have ionizable groups near the physiological range (pKₐ = 7.5 and 8.5, respectively). Thus, we have used variations in pH as an approach to investigate the interactions of dTTP and 5'-AdThd with thymidine kinase in purified preparations and intact cells. We have addressed the following questions: (a) how does pH affect the inhibition of thymidine kinase activity by dTTP? (b) how does pH affect the interaction of 5'-AdThd with the active (competitive) or the regulatory (antagonism) site of the enzyme? (c) how does pH affect the modulation of the uptake of dThd by 5'-AdThd in intact 647V cells? It is of interest to elucidate the mechanism of action of 5'-AdThd since it represents a novel class of drugs (16) with potential chemotherapeutic applications. Furthermore, observations in intact cells could provide a new lead for the exploitation of differences in pH between tumor and normal cells.

In this paper we report that inhibition of purified thymidine kinase from 647V cells by dTTP is pH dependent. However, neither the competitive inhibition of the enzyme nor the antagonism of the inhibitory effects of dTTP by 5'-AdThd are pH sensitive. In intact cells exposed to lower pH media (6.5), the uptake of thymidine was less but it was more potently stimulated by 5'-AdThd than at pH of 7.5 or 8.5. The observations are consistent with a greater degree of inhibition of the enzyme (in vitro and in vivo) at the lower pH.

MATERIALS AND METHODS

Reagents. The [8-¹H]dATP (20 Ci/mmol) and [methyl-¹H]dThd (70 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). 5'-AdThd and dThd were purchased from Sigma Chemical Co. (St. Louis, MO). dTTP was obtained from P. L. Biochemicals (Milwaukee, WI). Alamine (tri-n-octylamine) was obtained from Aldrich Chemical Co. (Milwaukee, WI), and the Freon (1,1,2-trichloro-1,2,2-trifluoroethane) was from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other materials were obtained from Sigma Chemical Co., unless otherwise specified.

Cell Culture. The culture of 647V cells (17) has been described before in intact cells exposed to lower pH media (6.5), the uptake of thymidine was less but it was more potently stimulated by 5'-AdThd than at pH of 7.5 or 8.5. The observations are consistent with a greater degree of inhibition of the enzyme (in vitro and in vivo) at the lower pH.
Thymidine Kinase Purification and Assay. We purified thymidine kinase from 647V cells by affinity column chromatography by using the methodology previously described (9, 10). Thymidine kinase activity was measured (37°C) by using 3 μM dThd as indicated before (9, 10), except that the reaction mixture contained different buffers at different pH conditions (as indicated in the figure legends).

Nucleoside Uptake. The experiments were done 2 days after plating the cells at 1 x 10⁶ cells in 60-mm dishes (grown in minimal essential medium supplemented with 10% fetal bovine serum). The cells were incubated for 1 h in minimal essential medium buffered at pH 6.5 (25 mM morpholinooctanesulfonic acid), 7.5 (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), or 8.5 (25 mM Tris) at 37°C. Then the cells were exposed to 3 μM [³H]dThd in the presence of various concentrations of 5’-AdThd for 1 h. After aspirating the media, the cells were washed three times with ice-cold phosphate-buffered saline and then treated with 0.5 N HClO₄ for 30 min at 4°C. A portion of the acid-soluble fraction was counted in ACS (Amersham Corp., Arlington Heights, IL) in a liquid scintillation spectrometer.

Measurement of Intracellular pH. We quantified the perturbation of the intracellular pH as a function of the extracellular pH by using the flow cytometric methodology described by Alabaster et al. (18). Briefly, 647V cells were incubated in rotating cultures for about 2 h. Then, the cells were incubated for 1 or 2 h in test medium buffered at the appropriate pH with morpholinooctanesulfonic acid (pH 6.0 and 6.5), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0–8.0), or Tris (pH 8.5 and 9.0). The cells were centrifuged and resuspended in 1 ml medium (3–5 x 10⁶ cells); 20 μl of ADB (in 1 mg/ml dimethylformamide) were added. ADB is then converted intracellularly by nonspecific esterases to a compound with a fluorescence emission spectrum which is pH dependent. The standard curves were obtained by incubating the cells with ADB and 20 μl of ammonium acetate (1 M) in media buffered at various pH values (6–9). Then, the cells were incubated at room temperature for 5 min. Analysis was done by using a Coulter flow cytometer coupled to a multiple data acquisition and display system and to an electrically programmable individual cell sorter. An argon ion laser emitted 180 mw of UV at 351 and 361 nm. The fluorescence emission spectrum was monitored simultaneously at two different wavelengths: 405 ± 20 nm and 550 ± 20 nm. Linear regression analysis was done to extrapolate the intracellular pH values from the plot of the ratio of the fluorescence at both wavelengths (405/550, as modal channel number) as a function of the pH of the standards. The correlation coefficient of the straight line fit was 0.980.

Measurement of dTTP Pools. The cells were plated at 1 x 10⁶ cells in 100-mm dishes. After 2 days the cells were treated as detailed in the text. The intracellular dTTP pools were extracted as previously described (10) and quantitated by using a DNA polymerase assay (19).

Synthesis and Purification of [³H]5’-AdThd. The synthesis of [³H]5’-AdThd from [³H]dThd was accomplished by a modification of the procedure described by Lin and Prusoff (20). Five mCi of [³H]dThd were evaporated to dryness, then coevaporated twice with 2 ml of dry pyridine (Aldrich) in vacuo and stored overnight in a desiccator under vacuum. dThd was dissolved in 0.5 ml dry pyridine and cooled to 0°C; 20 mg of p-toluenesulfonyl chloride (Aldrich) were then added and the mixture was incubated overnight at 4°C in a desiccator. Distilled water was added to the reaction mixture and evaporated to dryness in vacuo. The residue was dissolved in 1 ml of dimethyl formamide (Aldrich) and 20 mg of lithium azide (Kodak). The mixture was heated for 2 h at 75°C, then evaporated to dryness in vacuo. The mixture was resuspended in 1 ml of 50% ethanol; 1 mg of palladium black (Sigma) was added. Hydrogenation was done for 4 h in a Parr hydrogenator under 60 psi. The mixture was then centrifuged and the supernatant was stored at -20°C. Purification of [³H]5’-AdThd was achieved by using a two-step liquid chromatography process. First, dThd and 5’-AdThd were separated from other reaction components by HPLC on a Beckman Ultrasil-ODS column eluted with 0.1% acetic acid (2 ml/min). The radioactive eluting between 18 and 35 min was collected (retention times for 5’-AdThd and dThd are 26.5 and 29 min, respectively). Separation of 5’-AdThd from dThd was accomplished by cation exchange chromatography by using 1-ml Dowex-50 cation exchange columns previously equilibrated with water. The mixture was loaded and dThd was eluted with water. 5’-AdThd was eluted with 25 N ammonium hydroxide and then neutralized with HCl. The material is concentrated in vacuo. The purity and identity of the final product was verified by reverse phase HPLC (Beckman Ultrasil-ODS) eluted with 0.5% acetic acid. At 2 ml/min, the retention times for 5’-AdThd and dThd are 14.5 and 21 min, respectively. The overall yield of the procedure is approximately 10%.

Determination of Intracellular Accumulation of 5’-AdThd. To determine the uptake and accumulation of 5’-AdThd as a function of pH, we incubated the cells at 37°C in the different buffered media for 1 h. The cells were then exposed to 3 μM [³H]5’-AdThd. After the appropriate time period, the medium was rapidly aspirated and the plates were quickly immersed in a series of three ice-cold washes of 5 μM diprydamole (in phosphate-buffered saline) to stop the efflux of the drug (21). The cells were then treated with 0.5 N HClO₄ for 30 min and the acid-soluble fraction was processed as described above for nucleoside uptake. A portion was counted in ACS. Verification of the identity of the extracted species as 5’-AdThd was made by HPLC as described above.

Determination of Nucleoside Phosphorylase Activity. We determined the breakdown of thymidine to thymine by using conditions similar to the nucleoside uptake experiments described above. The procedure has been detailed before (11).

RESULTS

Effect of pH on Inhibition of Thymidine Kinase Activity by dTTP. Thymidine kinase purified by affinity column chromatography from 647V cells was used in these experiments. We investigated the effect of pH on inhibition of thymidine kinase activity by dTTP (Fig. 1). The enzyme showed a broad peak of activity with a pH optimum of about 8.0. The interaction of dTTP, which has a 50% inhibitory concentration of 1.8 μM (8), at the regulatory site of thymidine kinase was highly sensitive to variations in pH. Fluctuations of pH around the pKa value of dTTP (≈7.5) greatly affected the ability of dTTP to inhibit the enzyme. For example, 2.5 μM dTTP inhibited enzyme activity 50, 75, and 90% at pH of 8.0, 7.5, and 7.0, respectively. Thus, lower pH conditions result in a more potent inhibition by dTTP.

Effect of pH on Interactions between Thymidine Kinase and 5’-AdThd. 5’-AdThd interacts with thymidine kinase at both the active and the regulatory sites. Interactions at the active site result in competitive type inhibition with dThd, with a 50% inhibitory concentration of 1.8 μM (8). In the presence of dTTP,
interactions of 5'-AdThd at the regulatory site result in antagonism of the dTTP-induced inhibition of enzyme activity. Two observations lead to study pH as a possible modulator of the different interactions of 5'-AdThd with the enzyme: (a) the 5'-amino group of 5'-AdThd has a pKₐ value of 8.5, and (b) pH significantly affects the interaction of dTTP at the regulatory site (see above).

We studied the effect of pH on the competitive inhibition of thymidine kinase activity by 5'-AdThd (active site effect) by using 3 μM dThd (Kᵣ = 1.8 μM) as the substrate. Varying the pH of the assay mixture from 6.5 to 9.5 had no effect on the inhibition of enzyme activity by 5'-AdThd (Fig. 2).

To study the effect of pH on the ability of 5'-AdThd to stimulate (deinhibit) thymidine kinase activity in the presence of dTTP, we varied the concentrations of dTTP for the various pH conditions to obtain a similar degree of inhibition of enzyme activity. Thymidine kinase activity was inhibited to about 15% in the presence of 0.7, 3, 9, and 13 μM dTTP at pH conditions of 6.5, 7.5, 8.5, and 9.5, respectively. Under these conditions, the ability of 5'-AdThd to partially reverse the dTTP-induced inhibition of thymidine kinase activity was independent of pH (Fig. 3).

To more closely parallel the regulation of the enzyme in intact cells, we examined the effect of pH on the deinhibition of thymidine kinase by 5'-AdThd at a fixed concentration of dTTP. Based on our findings (see above) we would expect that at a lower pH of the reaction mixture there should be: (a) a higher inhibition of enzyme activity by dTTP; (b) a greater degree of antagonism by 5'-AdThd; and (c) a larger concentration of 5'-AdThd should be required to achieve maximal stimulation of thymidine kinase activity. In fact these were seen. At 2 μM, dTTP inhibited enzyme activity 95, 85, and 75% of control at pH conditions of 6.5, 7.5, and 8.5, respectively. Under these conditions, 5'-AdThd stimulated (maximum) thymidine kinase activity 230, 146, and 115% relative to control (no 5'-AdThd); the optimal concentrations of 5'-AdThd required were 30, 3, and 1 μM, at pH values of 6.5, 7.5, and 8.5, respectively. Higher concentrations of dTTP were also studied but essentially complete inhibition of enzyme activity occurred at pH 6.5 (data not shown).

Effect of pH on Modulation of dThd Uptake by 5'-AdThd in 647V Cells. We have previously shown that 5'-AdThd stimulates the uptake of dThd and IdUrd in 647V cells (9, 10). We investigated the effect of pH on the ability of 5'-AdThd to perturb thymidine uptake. To modulate the intracellular pH, we exposed cells to media buffered at various pH values following the methodology described by Kennedy et al. (22), in which reasonable changes in intracellular pH were obtained at different extracellular pH conditions. 647V cells were exposed for 1 or 2 h to media buffered at pH 6.5, 7.5, and 8.5. This treatment resulted in intracellular pH values of 7.00, 7.35, and 7.68 after 1 h and of 6.98, 7.21, and 7.61 after 2 h, respectively (data not shown). The uptake of 3 μM [3H]dThd was then determined in the presence of various concentrations of 5'-AdThd after 1 h. According to the results presented above we would expect that: (a) the total amount of dThd uptake should increase with increasing pH, since the intracellular dTTP should be less inhibitory to thymidine kinase; and (b) at lower pH conditions, a greater degree of stimulation of dThd uptake at higher concentrations of 5'-AdThd should result. In fact, these were seen. The uptake of dThd was 150, 180, and 200 pmol/10⁶ cells at pH of 6.5, 7.5, and 8.5, respectively (Fig. 4). Stimulation of
In this paper we have verified the pH dependence of the inhibition of thymidine kinase activity by dTTP (Fig. 1) by using enzyme purified by affinity chromatography from 647V cells. This was previously done with a crude preparation of enzyme extracted from Ehrlich ascites tumor cells (15). It should be noted that the changes observed occurred around pH 7.5, which corresponds to the pK_a of dTTP. This suggests that the protonation of the β-phosphate group results in greater affinity of dTTP to the regulatory site and greater inhibition of enzyme activity. The interaction of 5'-AdThd (pK_a = 8.5) with thymidine kinase (either active or regulatory site) was not significantly affected by pH (Figs. 2 and 3). The nature of the interaction at the regulatory site presents an interesting phenomenon because dTTP and 5'-AdThd have opposite charges near the physiological pH range and both have different effects on enzyme activity. However, appropriate binding studies would be required to directly examine this interesting interaction.

An important contribution from our study is the demonstration that pH modulates the intracellular regulation of thymidine kinase by dTTP. Perturbation of the intracellular pH was accomplished by incubating the cells in media buffered at different pH values. This was corroborated by measurement of intracellular pH with a pH-sensitive fluorochrome (ADB) and flow cytometric techniques. The results obtained from the dThd uptake experiments (Fig. 4) are consistent with perturbation of thymidine kinase activity in situ by dTTP. This is evident when compared with the results obtained in vitro by using a constant concentration of dTTP (see “Results”). In both cases, the ability of 5'-AdThd to antagonize the feedback inhibitory effects of dTTP was independent of pH (Fig. 3) but dependent on the degree of inhibition exerted by dTTP. The biphasic nature of the curves in Fig. 4, as indicated before (10), is due to the net effect of 5'-AdThd on thymidine kinase activity. At low concentrations and in the presence of dTTP, 5'-AdThd deinhibits thymidine kinase thus resulting in increased activity. In the absence of dTTP or at high concentrations of 5'-AdThd, interactions at the active site predominate, thus resulting in competitive inhibition of enzyme activity by 5'-AdThd.

We have used 5'-AdThd to perturb the regulation of thymidine kinase in situ, resulting in the demonstration of the pH dependence of the regulation of the enzyme. This approach differs from studies of the in situ regulation of the mammalian CTP synthetase (24), in which mutants were used, or of the bacterial citrate synthase (25), in which recombinant DNA techniques were used. In our case we have used direct modulation of the enzyme by an effector molecule.

The sensitivity of enzymes to pH is well known. Protonation of important amino acid residues result in characteristic pH profiles of enzyme activity (26). pH can also affect the allosteric [e.g., tryptophan oxygenase (27), AMP deaminase (28), and pyruvate kinase (29)] or the hysteretic behavior [e.g., β-glucosyltransferase (30, 31) and ovoperoxidase (32)] of enzymes. In fact, Gregory and Ainsworth (33) have described a method for the analysis of kinetic data for regulatory enzymes as a function of pH.

The ionization of substrates or other effector molecules (e.g., inhibitors, activators) is also of importance in considering pH effects on enzyme activity. Trivedi and Danforth (34) showed that the activity and regulation of frog muscle phosphofructokinase is sensitive to variations in pH in the physiological range. They found that the effects of ATP (inhibition), fructose 6-phosphate or 5'-AMP (activators) on enzyme activity are pH dependent. Pyruvate kinase from yeast and rat liver show dependence on the interaction between enzyme and the effector molecule, fructose 1,6-diphosphate (35, 36). Another example...
is the demonstration by Kessel that deoxycytidine kinase derived from L1210 cells is more inhibited by dCTP at lower pH conditions (37).

We have demonstrated the dependence of the regulation of the in situ form of thymidine kinase on the intracellular pH. That enzyme activity in intact cells can be affected by extracellular pH has been shown before for enzymes of the glycolytic pathway such as hexokinase (38) and phosphofructokinase (39). The importance of extracellular pH for cell growth is well known (40-42). In our study we have shown the pH dependence of the uptake of thymidine by 647V cells. As reported before (43, 44), the uptake of thymidine decreases with decreasing pH of the extracellular media. Here we have shown that this could be due to a greater inhibition of thymidine kinase by dTTP.

This report could be of importance for studies in tumor biology. It has been shown that some chemical carcinogens, oncogenes, and growth factors can alter intracellular pH in various cellular systems (45-47). Based on our study, changes in the intracellular pH as a result of exposure to these agents could potentially affect the regulation of the activity of important enzymes and, thus, of metabolic pathways.

The demonstration that the in situ regulation of thymidine kinase activity by dTTP is sensitive to pH has important implications for chemotherapy since this enzyme is responsible for the activation of several anticancer and antiviral agents. In various studies, a difference in pH between tumor and normal tissue has been demonstrated (48, 49). Usually tumor pH is more acidic. Others have attempted to maximize these differences by using glucose and breathing of a non toxic CO2-air mixture in mice (50). Furthermore, various groups have evaluated the effect of pH on the antitumor activity of chemotherapeutic agents (51), heat (52), or radiation (53). In fact, it has been proposed to develop a new class of anticancer drugs to interfere with the regulation of intracellular pH (54).

Two recent reports provide new insights on the results reported in this paper. We have recently shown (55) that the regulation of thymidine kinase activity can differ among various cell types. Two cell types (normal human urothelial and mouse C3H/10T½/5) in which dThd uptake was only marginally increased by 5'-AdThd at pH 7.5, showed substantial perturbation of dThd uptake by 5'-AdThd when incubated at pH 6.5. Interestingly, the enzyme isolated from these cells were similar in responsiveness to 5'-AdThd as the thymidine kinase derived from 647V cells. In another report (56), evidence was provided for the ability of 5'-AdThd to enhance the incorporation of IdUrd into DNA of 647V cells. This effect was also dependent on pH. The enhancement of the incorporation of IdUrd into DNA resulted in an increase in radiosensitization.

Our study opens a new route of investigation by considering differences in pH as a potential tool for therapeutic exploitation based on differences in the intracellular regulation of key activating enzymes.

ACKNOWLEDGMENTS

We wish to thank Eric Hanson for his expert technical assistance in performing the determination of intracellular pH with the fluorescence-activated cell sorter. We also acknowledge the aid of Mary Pankratz in the preparation of this manuscript.

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