Human Serum Thymidine Triphosphate Nucleotidohydrolase: Purification and Properties of a New Enzyme†

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ABSTRACT: Thymidine triphosphate monophosphohydrolase (dTTPase), an enzyme which catalyzes the hydrolysis of dTTP to the corresponding diphosphate (dTDP), has been purified to homogeneity from human serum. The enzyme sediments with 3.8 S in sucrose density gradients. A Stokes radius of 31 Å is estimated by gel filtration. Accordingly, its molecular weight is 48 500. Since only one single band of M_r 24 000 is detected after sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the native enzyme seems to be composed of two identical subunits. The enzyme exhibits optimal activity over a pH range from 7 to 9, and the activation energy is estimated to be 7.1 kcal/mol (29.7 kJ/mol) at pH 7.8. While the enzyme is active in the absence of added divalent cations, the activity can be inhibited by ethylenediaminetetracetic acid (EDTA) but not by phenanthroline. The inhibition caused by EDTA is reversed by Mn^{2+}. Zn^{2+} causes a complete inhibition of enzyme activity. No requirement exists for a sulfhydryl compound. The enzyme has an R_f value of 0.45, an isoelectric point of 5.2, and an apparent K_m value of 40 μM for dTTP. dUTP and UTP are degraded by about 50 and 20% of the rate of dTTP hydrolysis, respectively. Other deoxyribonucleosides or ribonucleoside triphosphates do not serve as substrates for the dTTPase. The existence of this enzyme is significant since it could play a role in the regulation of the cellular dTTP levels.

The presence of all four deoxyribonucleoside triphosphates (dNTP) is required for DNA synthesis to proceed at an appreciable rate. In eucaryotes the synthesis occurs at the diphosphate level catalyzed by the enzyme ribonucleotide reductase. The deoxyribonucleoside diphosphates so formed are phosphorylated to the corresponding triphosphates. The concentrations of all four dNTPs seem to be under a stringent control, which is partly exerted by the dNTPs themselves and partly by ATP through feedback regulation of several steps involved in dNTP metabolism (Bjursell & Skoog, 1980). One step is the control of the ribonucleotide reductase by several allosteric effectors. The compounds ATP and dTTP act as positive allosteric effectors for the reduction of the pyrimidine nucleoside diphosphates. However, in the presence of ATP, low concentrations of dTTP stimulate the reduction of pyrimidine ribonucleotides, whereas larger amounts are inhibitory (Larsson & Reichard, 1966a,b). Therefore cells incubated with high concentrations of the dThd are characterized by a large expansion of the intracellular dTTP pool and a depletion of cellular dCTP (Bjursell & Reichard, 1973) and dATP levels (Tattersall et al., 1975) as a consequence of an inhibition of the ribonucleotide reductase. These findings suggest that the level of the dTTP itself must be regulated.

In this context it was of special interest that in a preliminary study (Dahlmann & Müller, 1980) an enzymatic activity was described catalyzing the hydrolysis of dTTP to dTDP. The present report describes the purification of human serum thymidine triphosphate nucleotidohydrolase (dTTPase) to homogeneity and its characterization with respect to its substrate specificity, electrophoretic behavior, molecular weight, activation energy, and kinetic properties.

Materials and Methods

Substrates. All nucleotides were obtained from Boehringer-Mannheim. Dithiothreitol was purchased from Sigma Chemical Co., and Polygram CEL 300 PEI, precoated sheets, were supplied by Macherey-Nagel Co. Other standard reagents were purchased from E. Merck. All chemicals utilized were of the highest quality commercially available.

Chromatography Supports. DEAE-cellulose, DE-52, was purchased from Whatman, hydroxylapatite Bio-Gel HT from Bio-Rad, phosphocellulose, Servacel P, from Serva, DEAE Affi-Gel Blue from Bio-Rad, and Sephacryl S-200 from Pharmacia.

Labeled Nucleotides. [8-^{3}H]dATP, [5-^{3}H]dCTP, [8-^{3}H]-dGTP, [methyl-^{3}H]dTTP, [5-^{3}H]dUTP, [8-^{3}H]ATP [5-^{3}H]-CTP, [8-^{3}H]GTP, and [5-^{3}H]UTP were all purchased from Amersham Buchler.

Serum. Serum of a healthy blood donor no. 90 88 154 was obtained from Dr. B. v. Eisenhardt-Rothe, Chirugische Universitätsklinik, Hamburg.

dTTPase Assays. Two different assays were developed. *Assay A*, a semiquantitative screening assay, was used for the purification procedure and the characterization. Each assay mixture contained, in a final volume of 50 μL, 10 mM Tris-HCl[1] (pH 7.8), 1 mM $MnCl_2$, 8 mM dithiothreitol, 6 μM [^{3}H]dTTP (7–8 μCi/nmol), and 0.2 mg/mL bovine serum albumin. The enzyme samples were diluted to give 20% hydrolysis of the substrate. *Assay B* is a quantitative assay and the same as assay A except for the substrate concentration of 51 μM [^{3}H]dTTP (0.8 μCi/nmol) sustaining 58% of the V_{max} value. Incubation was at 30 °C for 1 h unless otherwise indicated. One unit of activity represents the production of 1 pmol of dTDP/h.

The reaction was terminated by the addition of 5 μL of 50% (v/v) formic acid containing unlabeled tri-, di-, and monophosphates of the respective substrates. The reaction mixtures were immediately centrifuged (2 min at 10000*g*), and 5-μL aliquot portions of the supernatant were applied to PEI–cellulose sheets. The chromatograms were developed with 1 M acetic acid/0.85 M LiCl for the substrates dTTP, dUTP, and

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PEI, poly(ethylenimine); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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Table I: Purification of dTTPase Activity from Human Serum

procedure	volume (mL)	total units ($\times 10^{-3}$)[a]	total protein (mg)	sp act. (units/mg)	yield (%)	purification (fold)
(1) human serum	200	3700[b]	15600	237	100	1
(2) ammonium sulfate (30-60%)	150	1970[b]	4300	458	53	2
(3) DEAE-cellulose chromatography	305	792[b]	460	1722	21	7
(4) hydroxylapatite chromatography	90	600	70	8530	16	36
(5) phosphocellulose chromatography	48	383	26	14730	10	62
(6) DEAE Affi-Gel Blue	2.2[c]	231	9	25670	6	108
(7) isoelectric focusing		231[d]	1.3	177690	6	750

[a] Values based on assays at 51 μM dTTP (see Materials and Methods) are 58% of the V_{max} value. [b] The enzymatic activity in step 1 and step 2 is divided by four and in step 3 by two according to unspecific activities (Dahlmann & Ueckermann, 1982). [c] Volume after concentration. [d] Calculated under the prediction of 100% recovery from step 6.

UTP and 2 N formic acid/0.5 M LiCl for the substrates dATP, dCTP, dGTP, and the corresponding ribonucleotides at room temperature, dried, and examined with UV light. The spots were each cut out and radioactivities determined by liquid scintillation counting without prior elution.

Protein Assay. Protein was assayed by the procedure of Lowry et al. (1951).

Sucrose Gradient Sedimentation. Sedimentation analysis of purified dTTPase was carried out by cosedimentation with bovine immunoglobulin G, hemoglobin, and cytochrome *c*, in a linear (4.8 mL), 5–20% (w/v) sucrose gradient (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 5 mM dithiothreitol), in a SW 65 Ti rotor at 63 000 rpm, 4 °C, for 16.5 h.

Determination of Stokes' Radius. Stokes' radius was determined as described by Siegel & Monty (1966) by using a Sephacryl S-200 column (0.7 × 30 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.8) and 50 mM NaCl and eluted with the same buffer at a flow rate of 1.3 mL/h collecting 0.38-mL fractions. The void volume, V_0, was determined from the elution volume of dextran blue 2000 and the total volume, V_t, from a calibration of the empty column with water. Results were plotted as $(-\log K_{av})^{1/2}$ vs. Stokes' radius, where $K_{av} = (V_e - V_0)/(V_t - V_0)$.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was conducted in 12.5 or 15% polyacrylamide gels [1:40 concentration of bis(acrylamide)] with 0.1% NaDodSO$_4$ in a Tris/glycine buffer system (Laemmli, 1970). Gels were stained with Coomassie brilliant blue. Protein samples (8/20 μg) for NaDodSO$_4$ gel electrophoresis were reduced in 2% NaDodSO$_4$ and 2% mercaptoethanol and briefly heated at 100 °C in sample solution (Kruppa, 1979), prior to electrophoresis. Electrophoresis was carried out at 30 mA at 4 °C until the bromphenol blue dye marker reached the bottom of the gel.

Analytical Disc Gel Electrophoresis. Disc electrophoresis was carried out in tubes of 4 mm width in a 7.5% polyacrylamide gel [1:40 concentration of bis(acrylamide)] in 0.25 M Tris-HCl buffer (pH 8.9). Fifty-microliter samples containing 30 μg of dTTPase were layered on the top of the tubes, using bromphenol blue as a marker. The electrophoresis was performed at 4 °C with a constant current of 2.5 mA/tube applied across the gel for approximately 3.5 h. The running buffer contained 50 mM Tris/glycine (pH 8.4). At the end of the run, gels were removed from the tubes and sliced in 4-mm pieces. Each piece was immersed in 150 μL of the reaction mixture (see assay A) but without the substrate and incubated overnight at 4 °C, and the supernatant was assayed as described.

Electrofocusing. Isoelectric focusing was performed in polyacrylamide gel rods of 4 mm width using Ampholine 3.5–10. The gel was polymerized from acrylamide (7.5%), bis(acrylamide) (0.2%), ammonium persulfate (0.2%), and *N,N,N',N'*-tetramethylethylenediamine. Samples (100 μL containing 59 μg of the dTTPase) were layered on the top of the tubes. The electrophoresis was performed with a Pharmacia apparatus at 4 °C with 1–2 mA/gel rod, setting a maximum voltage of 800 V. After about 6 h gels were removed from the tubes and sliced in 4-mm fractions for elution and assay as described above. For the second dimension the sliced and homogenized gel pieces were immersed in a sample solution (Kruppa, 1979), heated at 100 °C, and applied to a 15% NaDodSO$_4$/polyacrylamide gel.

NDP Kinase Assay. The reaction was performed under the conditions described for assay A. After 1 h of incubation at 30 °C, ATP at a final concentration of 50 μM was added, and incubation was continued for another 2 h at 30 °C. Samples were taken at 0, 15, 30, 60, and 120 min after the addition of ATP and assayed as described above.

Gel Scanning. A sample of the purified enzyme (step 6) was analyzed on a NaDodSO$_4$/polyacrylamide gel. The stained sample lane was photographed with a Polaroid camera, and the negative was scanned with a Beckman Model DU-8 spectrophotometer at 420 nm. The peaks of the scan were cut out and analyzed by gravimetry. The amount of dTTPase is expressed as percent of the total protein.

Results

Purification of dTTPase. Enzymatic activity was monitored by following the decrease in the amount of [^{3}H]dTTP. The only product of dTTP hydrolysis by dTTPase as identified by thin-layer chromatography was dTDP. The purification is summarized in Table I.

Human Serum. The enzyme was purified from a 200-mL sample of human serum.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added slowly to 200 mL of serum to give 30% saturation; the resulting solution was stirred for 60 min at 0 °C, and the precipitate that formed was removed by centrifugation at 12000*g* for 15 min. Solid ammonium sulfate was again added to the supernatant fraction (200 mL), containing about 90% of the total dTTP hydrolyzing activity, to yield 60% saturation. The precipitate was suspended in $^1/_6$ volume of 20 mM potassium phosphate buffer (pH 6.4) containing 5 mM mercaptoethanol (buffer A), and the solution was dialyzed overnight against 40 volumes of the same buffer.

DEAE-cellulose Chromatography. The above dialysate was applied to a 200-mL DE-52 column (2.5 × 44 cm) preequilibrated with buffer A. The column was washed with buffer A and eluted with buffer A containing 50 mM NaCl (buffer B). Further elution was carried out with a linear gradient of buffer B (400 mL) and buffer A containing 200 mM NaCl (buffer C) (400 mL). Protein elution was monitored by absorbance at 280 nm, and the gradient was measured by conductivity. The dTTPase activity was eluted as a broad peak

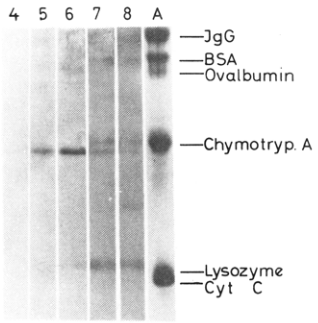

FIGURE 1: $NaDodSO_4$/polyacrylamide gel electrophoresis as the second dimension of isoelectric focusing. The numbers of the lanes are identical with fraction numbers of the isoelectric focusing (see Figure 3B). The sliced and homogenized gel pieces of the isoelectric focusing were applied to a 15% $NaDodSO_4$/polyacrylamide gel. Protein standards and molecular weights (lane A) were 8–20 μg of bovine immunoglobulin G (150 000), bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000), and cytochrome *c* (12 300).

at a NaCl concentration of 120 mM. The content of the tubes with maximal enzyme activity was collected, pooled, and dialyzed for 12 h against 40 volumes of 100 mM potassium phosphate (pH 6.4) containing 5 mM mercaptoethanol (buffer D).

Hydroxylapatite Chromatography. The above dialysate was loaded on a 54-mL Bio-Gel HT column (2.5 × 11 cm) preequilibrated with buffer D. Elution was obtained with a linear gradient of buffer D (150 mL) and buffer E [250 mM potassium phosphate (pH 6.4) containing 5 mM mercaptoethanol] (150 mL). The retained enzyme was eluted when the buffer concentration reached 150 mM phosphate. Tubes corresponding to maximal enzyme activity were collected, pooled, and dialyzed for 12 h against 40 volumes of 20 mM potassium phosphate buffer (pH 5.3) containing 5 mM mercaptoethanol (buffer F).

Calcium Phosphate–Cellulose Chromatography. The above dialysate was applied to a 12-mL calcium phosphate–cellulose column (1.5 × 7 cm), preequilibrated with buffer F. The column was washed with buffer F, and elution was carried out with a linear gradient of buffer F (40 mL) and buffer G [200 mM potassium phosphate (pH 5.3) containing 5 mM mercaptoethanol] (40 mL). The dTTPase activity was retained on the column and eluted when the buffer concentration reached 70 mM phosphate. Tubes corresponding to maximal enzyme activity were collected, pooled, and dialyzed for 12 h against 40 volumes of 10 mM potassium phosphate buffer (pH 8.1) containing 5 mM mercaptoethanol (buffer H).

DEAE Affi-Gel Blue Chromatography. The above dialysate was loaded on a 1.2-mL DEAE Affi-Gel Blue column (0.7 × 3 cm), which was preequilibrated with buffer H. The column was washed with 4 column volumes of equilibration buffer and the 4 column volumes of buffer I [100 mM potassium phosphate (pH 8.1) containing 5 mM mercaptoethanol]. Active fractions were pooled and concentrated 4-fold by vacuum dialysis in collodion bags (Schleicher & Schuell) against 10 mM Tris-HCl (pH 7.8) containing 5 mM mercaptoethanol. Aliquots of 100 μL were stored at −80 °C at which the dTTPase was stable for at least 6 months. There was a loss of activity of 10–20% upon each cycle of freezing and thawing the enzyme samples.

Electrofocusing. An aliquot of 100 μL of the above dialysate was electrofocused in a pH gradient of pH 3.5–10.0. The sliced gel pieces were applied to a 15% $NaDodSO_4$/polyacrylamide gel, where fraction 5 and fraction 6 of the isoelectrofocusing revealed a single band in the second dimension (Figure 1). The dTTPase concentration in the enzyme solution (step 6 of the purification procedure) was estimated to be about 15% of the stained proteins by gel scanning so that the total amount of the dTTPase can be calculated as 1.3 mg.

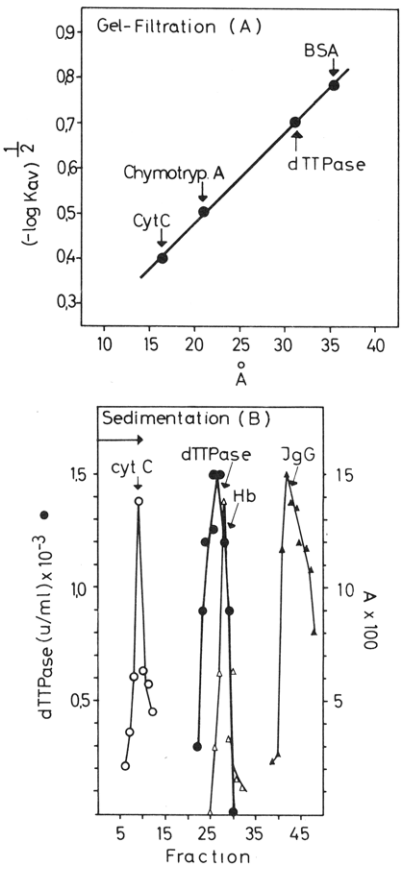

FIGURE 2: Determination of Stokes' radius and sedimentation coefficient of dTTPase. (A) Gel filtration of dTTPase (step 6) was carried out on a Sephacryl S-200 column, which was precalibrated with bovine serum albumin (35.5 Å), chymotrypsinogen A (20.9 Å), and cytochrome *c* (16.4 Å) as markers. (B) Sucrose gradient sedimentation of dTTPase was carried out by sedimentation of the enzyme with bovine immunoglobulin G, hemoglobin, and cytochrome *c*. Immunoglobulin G was assayed by absorbance at 280 nm, hemoglobin by absorbance at 430 nm, cytochrome *c* by absorbance at 550 nm, and dTTPase as described under Materials and Methods. The arrow indicates direction of sedimentation.

All experiments for characterization of the dTTPase were performed with the enzyme solution of step 6 in assay A except the determination of the substrate specificity and the product analysis of dTTP hydrolysis, which were carried out with fraction 6 of the isoelectrofocusing (step 7).

Molecular Weight and Subunit Structure. The molecular weight of dTTPase was determined by combining two procedures: gel filtration and sucrose density gradient centrifugation. The results shown in Figure 2A give a Stokes radius, r_s, of 31 Å and a sedimentation coefficient, $s_{20,w}$, of 3.8 S for dTTPase (Figure 2B). The molecular weight can be determined from

$$M_r = 6\pi r_s \eta N s_{20}/(1 - v_2\rho)$$

where N is Avogadro's number, v_2 is the partial specific volume (assumed to be 0.725 $cm^3\ g^{-1}$), and η is the viscosity of the medium (assumed to be 0.01002 $g\ s^{-1}\ cm^{-1}$) and ρ its density (0.99823 $g\ cm^{-3}$). From the above values a molecular weight of 48 500 was calculated and a frictional ratio (f/f_0) equal to 1.29, indicative of a somewhat nonspherical shape of the enzyme molecule. On the other hand, $NaDodSO_4$/polyacryl-

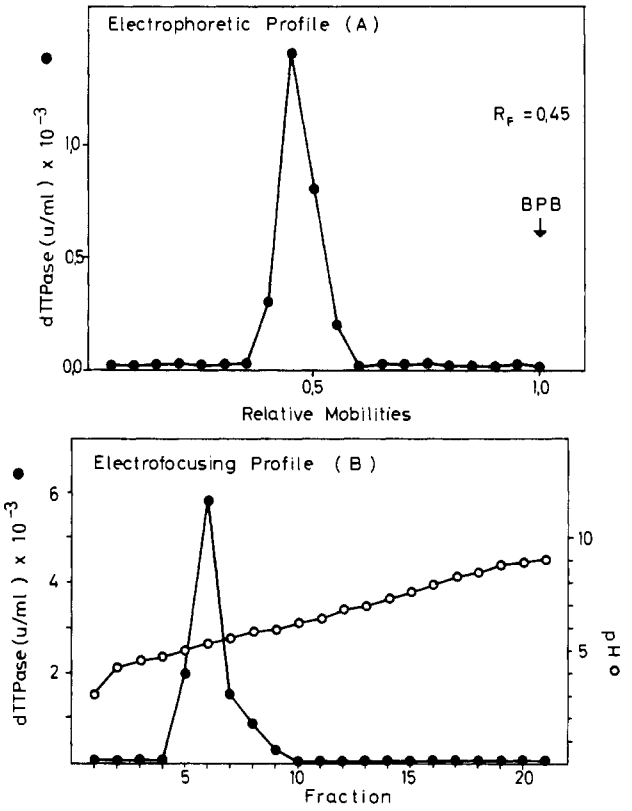

FIGURE 3: Electrophoretic and electrofocusing profile. The enzyme solution of step 6 of the purification procedure was used as the enzyme source. (A) Polyacrylamide gel electrophoresis was performed as described under Materials and Methods (30 μg of dTTPase). (B) Isoelectric focusing was performed in a pH gradient of pH 3.5–10. dTTPase (59 μg) was loaded on the gel rod. For the further procedure see Materials and Methods.

amide gel electrophoresis demonstrated that the enzyme protein migrated close to chymotrypsinogen A; the subunit polypeptide chain of dTTPase has an estimated molecular weight of 24 000.

Electrophoretic and Electrofocusing Profile. When electrophoresis was performed with the condition described above, the enzyme migrates as a single peak with an R_f value of 0.45 (Figure 3A). Isoelectric focusing of the dTTPase exhibited in a pH gradient (pH 3.5–10) an isoelectric point at pH 5.2 (Figure 3B).

Temperature Dependence of dTTPase Reaction. For determination of the activation energy for the dTTPase reaction, the enzyme was incubated at various temperatures (12.2–23.0 °C) for 1 h in the standard assay mixture A. In this temperature range, the enzyme was stable in the reaction mixture as verified by the linear relationship between the reaction product and time at the various temperatures. When the reaction velocity and absolute temperature were expressed as an Arrhenius plot, a linear curve was obtained (Figure 4). The activation energy was calculated to be 7.1 kcal/mol (29.7 kJ/mol). The stability of the dTTPase begins to decline at 30 °C and breaks down at 37 °C.

Substrate Specificity and Product Analysis. Radioactively labeled deoxyribonucleoside triphosphates and ribonucleoside triphosphates were treated with the enzyme solution at step 6 of the purification procedure for 30 and 60 min at 30 °C. The dTTPase showed a preferential activity for dTTP hydrolyzing dUTP and UTP in vitro to about 50 and 20% of the dTTP hydrolysis, respectively. The same results were obtained with fraction 5 and fraction 6 of the isoelectric focusing. No other deoxyribonucleoside or ribonucleoside triphosphate

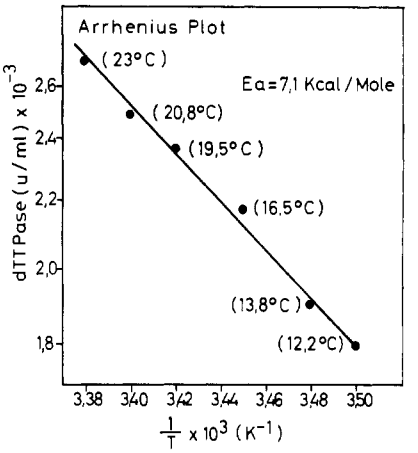

FIGURE 4: Arrhenius plots log v vs. $1/T$. Incubations were conducted at the temperature indicated. Incubation time was 30 min.

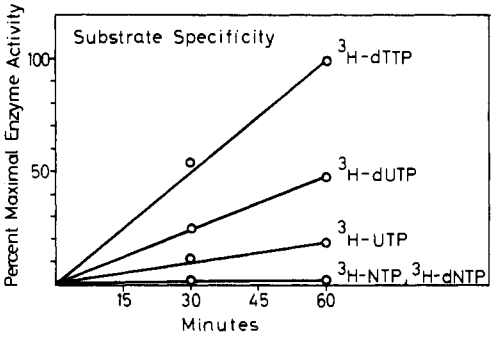

FIGURE 5: Substrate specificity. 50-μL assay solution contained at a substrate concentration of 51 μM in each case the following: [^{3}H]dTTP; [^{3}H]dUTP; [^{3}H]dNTP ([^{3}H]dATP, [^{3}H]dCTP, [^{3}H]-dGTP, [^{3}H]UTP); or [^{3}H]NTP ([^{3}H]ATP, [^{3}H]CTP, [^{3}H]GTP). Enzyme solution at step 6 of the purification procedure for 1 h at 30 °C was used. The amount of hydrolysis of dTTP to the diphosphate was taken as 100%.

served as a substrate for the dTTPase (Figure 5). The substrates dTTP, dUTP, and UTP are hydrolyzed to the corresponding diphosphates. The further degradation to the corresponding monophosphates is less than 2% in the isoelectric focusing preparation.

Enzymatic Properties of dTTPase. In Tris-HCl buffer, the enzyme has a broad pH optimum in the range 7.0–9.0. In the absence of any divalent cations the enzyme still retained enzymatic activity of about 70%. This activity was depressed by the addition of EDTA (0.2–2.0 mM) down to 20% but not by phenanthroline (0.2 mM). Of all divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) investigated to restore enzymatic activity, especially Mn^{2+} (1.0 mM) could reverse the inhibitory effect of EDTA. Except Zn^{2+}, they all exerted a bell-shaped curve in the concentration range 0.1–30 mM with an optimum at 1 mM. Of the divalent cations tested, Zn^{2+} ion showed in the absence of EDTA the greatest inhibition with 1.0 mM concentration bringing the activity down to zero.

The dTTPase (step 6 of the purification procedure) exhibited no requirement for a sulfhydryl compound. After thorough dialysis of the dTTPase against 10 mM Tris-HCl (pH 7.8), no loss of activity was observed in the absence of dithiothreitol. The addition of β-mercaptoethanol or dithiothreitol (10 mM) caused no activation. The kinetic analysis of dTTPase revealed that in the concentration range employed, dTTPase behaved as a Michaelis–Menten-type substrate with an apparent K_m value of 40 μM. This K_m value was the same when the crude preparation was subject to the kinetic analysis (Dahlmann & Ueckermann, 1982).

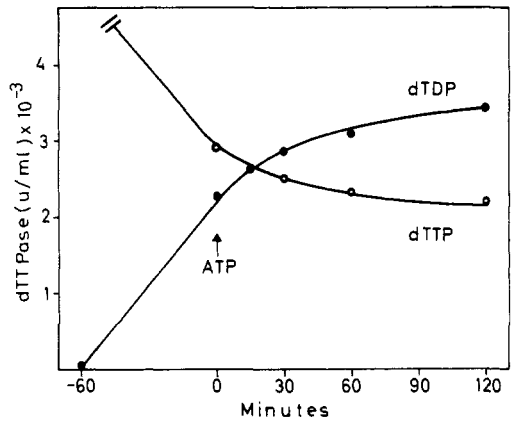

FIGURE 6: NDP kinase assay. After 1 h of preincubation, ATP at a final concentration of 50 μM was added, and incubation was continued for another 2 h.

The dTTPase showed no NDP kinase activity as can be seen from Figure 6. The preincubation was chosen because [^{3}H]dTDP is not commercially available. After this preincubation, the enzyme is still active, and no dTTP is formed in contrast to the crude preparation, where under the same assay conditions dTDP is immediately phosphorylated to the triphosphate.

Discussion

This paper describes the purification of thymidine triphosphate nucleotidohydrolase from human serum to near homogeneity and its characterization. In all analytical steps such as gel filtration, sedimentation, electrophoresis, and electrofocusing, only one single peak of activity could be detected for the purified dTTPase. The gel filtration technique used in this study pointed to a molecular mass for the dTTPase of 48 500 daltons using the Stokes radius and the sedimentation coefficient. By gel electrophoresis in sodium dodecyl sulfate a molecular mass of 24 000 daltons was calculated. It is therefore most likely that the native enzyme is composed of two identical subunits. Further the substrate specificity of the dTTPase observed in this study is generally coincident with an enzymatic activity previously found (Dahlmann & Müller, 1980). The enzyme has a preferential activity for dTTP producing dTDP exclusively. A further degradation is less than 2% in the homogeneous enzyme preparation. The reaction is not reversible even in the presence of excess ATP excluding the possibility that a back-reaction of NDP kinase is monitored.

With a serum level of approximately 100 μg/mL the concentration of the dTTPase seems to be relatively high; therefore we compared the amount of different enzymes in international units (IU) and the specific activity of the dTTPase with those of other key enzymes of the pyrimidine nucleotide metabolism. The dTTPase reveals an activity of 550 mIU/L serum taking into account a yield of 6% and a substrate concentration of 58% of V_{max}. This is a magnitude about 400–600-fold lower than the upper level of normal controls of enzymes in human serum like α-amylase, lactate dehydrogenase, and alkaline phosphatase so that there is no evidence that the dTTPase plays a biological role in human serum.

The comparison of the specific activity proves to be more difficult, because none of the key enzymes of pyrimidine nucleotide metabolism have been purified to homogeneity from mammalian sources. But the purification factors indicate near homogeneity so that a comparison may be allowed. Indeed, the specific activity of the dTTPase seems to be 5–10-fold lower than other key enzymes of pyrimidine nucleotide metabolism like thymidylate synthetase (Dolnick & Cheng, 1977), thymidylate kinase (Grav & Smellie, 1965), or cytidine triphosphate synthetase (McPartland & Weinfeld, 1976). This may be the reason that the protein concentration appears to be higher but of the same order of magnitude. Of course this calculation is done under the assumption that all detected protein of the dTTPase is still enzymatically active.

The unique characteristics of the dTTPase as described in this study make it very likely that a novel enzyme has been found. The enzyme is not related to the dUTPase, which is found in Yoshida sarcoma cells (Arima et al., 1977) and in blast cells of patients with acute leukemia (Williams & Cheng, 1979). The dUTPase is a tetramer with an apparent K_m of 1 μM, activated by Mg^{2+} and Zn^{2+} and inhibited by Mn^{2+} and which is highly specific for dUTP producing dUMP and PP_i exclusively (Shlomai & Kornberg, 1978; Williams & Cheng, 1979). The enzyme is also not identical with serum phosphodiesterase because of its specificity for dTTP and the inability to degrade the diphosphate. Other enzymes which degrade dTTP and dUTP have been found in bacteria; however, these enzymes differ in several aspects. *Bacillus subtilis* bacteriophage ϕe induces thymidine and deoxyuridine-5′-triphosphatase activities, which reside in a single enzyme producing the respective monophosphates (Dunham & Price, 1974). Furthermore these enzyme activities show an absolute requirement for a sulfhydryl compound (in contrast to the human dTTPase).

To date the biological function of the dTTPase is not understood. The substrate specificity of this enzyme suggests, however, that it might be involved in the regulation of cellular dTTP levels and thus may play an important role in the control of DNA synthesis.

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References

Arima, T., Akiyoshi, H., & Fujii, S. (1977) *Cancer Res. 37*, 1598–1601.

Bjursell, G., & Reichard, P. (1973) *J. Biol. Chem. 243*, 3904–3909.

Bjursell, G., & Skoog, L. (1980) *Antibiot. Chemother. (Basel) 28*, 78–85.

Dahlmann, N., & Müller, D. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1851–1854.

Dahlmann, N., & Ueckermann, Chr. (1982) *Biochem. Int. 5*, 185–192.

Dolnick, B. J., & Cheng, Y. Ch. (1977) *J. Biol. Chem. 252*, 7697–7703.

Dunham, L. F., & Price, A. R. (1974) *Biochemistry 13*, 2667–2672.

Grav, H. J., & Smellie, R. M. S. (1965) *Biochem. J. 94*, 518–524.

Kruppa, J. (1979) *Biochem. J. 181*, 295–300.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–682.

Larsson, A., & Reichard, P. J. (1966a) *J. Biol. Chem. 241*, 2533–2539.

Larsson, A., & Reichard, P. J. (1966b) *J. Biol. Chem. 241*, 2540–2549.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McPartland, R. P., & Weinfeld, H. (1976) *J. Biol. Chem. 251*, 4372–4378.

Shlomai, J., & Kornberg, A. (1978) *J. Biol. Chem. 253*, 3305–3312.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Tattersall, M. H., Ganeshaguru, K., & Hoffbrand, A. V. (1975) *Biochem. Pharmacol. 24*, 1495–1498.
Williams, M. V., & Cheng, Y. (1979) *J. Biol. Chem. 254*, 2897–2901.

A Study of *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*: Chemical Modification of Histidine Residues†

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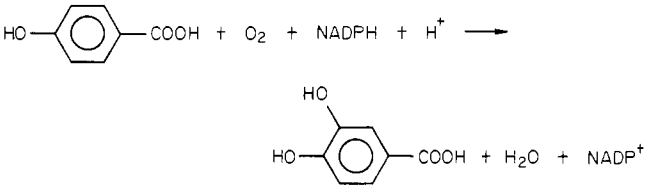
ABSTRACT: The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is inactivated by diethyl pyrocarbonate. Below pH 7, diethyl pyrocarbonate reacts specifically with histidine residues. The inactivation reaction is biphasic and follows pseudo-first-order kinetics. Four of the nine histidine residues of the enzyme are modified. During the first phase of the reaction, one histidine residue is modified and leads to a loss of about 30% of the activity. Modification of the additional three histidine residues during the second phase leads to complete loss of activity. Two of the latter histidine residues are essential for activity and are involved in the binding of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The activity can be restored almost quantitatively upon treatment of modified enzyme with hydroxylamine. The modified enzyme is still capable of binding NADPH. The dissociation constant of the enzyme–NADPH complex is larger by a factor of 10 for the modified enzyme as compared to that for the native enzyme. The modification does not affect the affinity of the enzyme for the substrate, although effectors protect two histidine residues from chemical modification by diethyl pyrocarbonate. The rate of inactivation of the enzyme is pH dependent and increases with increasing pH values. From the pH dependence of the rate constant, it is calculated that two cooperative histidine residues participate in the reaction with diethyl pyrocarbonate. Both histidine residues possess a pK_a' value of 6.2. At pH >7, other reactions take place which are completely abolished in the presence of an effector (substrate) of the enzyme.

The inducible enzyme *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) belongs to the class of external flavoprotein monooxygenases. It can be obtained from four different species of *Pseudomonas*, but the enzyme present in *Pseudomonas fluorescens* is the most stable one. It catalyzes the following reaction:



Significant progress has been made in recent years concerning the structure and catalytic mechanism of *p*-hydroxybenzoate hydroxylase (Shoun et al., 1979a; Müller et al., 1979; Husain & Massey, 1979). We have devoted ourselves to the study of the chemical modification of amino acid residues important for the catalytic activity of the enzyme. This is now a promising approach since the entire sequence of the enzyme is known (Hofsteenge et al., 1980; Vereijken et al., 1980; Weijer et al., 1982). In addition, the existing three-dimensional model of the enzyme–substrate complex at a resolution of 0.25 nm (Wierenga et al., 1979) allows a more detailed interpretation of the data of chemical modification. Furthermore, modification studies will yield results important for understanding the catalytic mechanism of the enzyme.

The work that has been done in the field of chemical modification so far includes arginine and histidine modification of the enzyme from *Pseudomonas desmolytica* (Shoun et al., 1979b, 1980) and cysteine modification of the enzyme from *P. fluorescens* (F. Müller and W. J. H. Van Berkel, unpublished experiments). Histidine modification by Shoun and his colleagues (Shoun et al., 1979b) was carried out by photooxidation at pH 6, and their conclusion was that a histidine residue with a pK_a' of 7.0 is involved in substrate binding by forming a hydrogen bridge with the phenolic OH of *p*-hydroxybenzoate. However, from the low-resolution three-dimensional model of the enzyme, it can be concluded that histidine is not involved directly in the binding of the substrate, at least not as far as the enzyme from *P. fluorescens* is concerned. This apparent discrepancy could be related to the fact that photooxidation reactions are not very specific, as tyrosine, methionine, and tryptophan can also be modified by photooxidation (Westhead, 1972).

The aim of this paper is to elucidate the role of histidine residues in *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. Our results clearly indicate that histidine residues are not involved in the binding of the substrate *p*-hydroxybenzoate, but rather in the binding of the pyridine nucleotide.

Materials and Methods

Diethyl pyrocarbonate was from Fluka, L-histidine monochloride was from the British Drug Houses Ltd., reduced nicotinamide adenine dinucleotide phosphate (NADPH)[1] was

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