8-Chloro-dGTP, a hypochlorous acid-modified nucleotide, is hydrolyzed by hMTH1, the human MutT homolog

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Abstract The human mutT homolog, hMTH1, suppresses spontaneous mutations by degrading the endogeneous mutagen, 8-hydroxy-dGTP. We previously reported the broad substrate specificity of hMTH1, which also degrades the oxidatively damaged purine nucleotides, 2-hydroxy-dATP, 8-hydroxy-dATP, 2-hydroxy-ATP, and 8-hydroxy-GTP, in addition to 8-hydroxy-dGTP. In this paper, we describe the hMTH1 activity for 8-chloro-dGTP, which could be formed in inflamed tissue by the reaction of dGTP with hypochlorous acid, a product of myeloperoxidase from activated human neutrophils. The hMTH1 protein was mixed with 1–20 μM of 8-chloro-dGTP and 8-hydroxy-dGTP, and the reaction products were quantified by anion-exchange HPLC to measure the pyrophosphatase reaction rate. The kinetic parameters revealed that 8-chloro-dGTP was degraded by hMTH1 with 50% efficiency as compared with that of 8-hydroxy-dGTP. This result suggests that 8-chloro-dGTP is an intrinsic substrate for hMTH1. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: 8-Chloro-dGTP; 8-Hydroxy-dGTP; hMTH1; *mutT*; Nucleotide sanitization enzyme

1. Introduction

Chronic infection by bacteria, parasites or viruses and tissue inflammation, such as gastritis and colitis, are recognized risk factors for human cancers at various sites [1]. Infection and inflammation activate a variety of inflammatory cells, which induce and activate various oxidant-generating enzymes. Activated human neutrophils secrete myeloperoxidase (MPO), which generates hypochlorous acid (HOCl) using hydrogen peroxide (H_2O_2) and chloride ion (Cl^-) as substrates. It has recently been reported that various chlorinated nucleosides, including the novel 8-chloro-2′-deoxyguanosine (8-Cl-dGuo), 5-chloro-2′-deoxycytidine, and 8-chloro-2′-deoxyadenosine, are formed by reactions of nucleosides with HOCl as well as with human MPO or activated human neutrophils in the presence of H_2O_2 and Cl^- [2–4]. Among the naturally occurring nucleosides, 2′-deoxyguanosine reacts with HOCl or human MPO to generate 8-Cl-dGuo with a high yield under physiological conditions, particularly in the presence of tertiary amines such as nicotine and trimethylamine, which act as catalysts to enhance the chlorination of nucleosides [2]. Although 8-Cl-dGuo has not been studied for mutagenicity, an analogous compound, 5-bromo-2′-deoxycytidine, is incorporated into DNA [5]. In addition, the incorporation of both dAMP and dCMP by human DNA polymerases occurred opposite the 8-bromo-2′-deoxyguanosine present in DNA templates, and induced G:C to T:A transversion mutations [6]. Thus, 8-Cl-dGuo is likely to be mutagenic, if it is incorporated or formed within DNA.

In the present study, as a prelude to an evaluation of the biological significance of this modified base, we have tested whether 8-Cl-dGTP, a 5′-triphosphate derivative of 8-Cl-Guo, is a substrate for the human enzyme hMTH1. This enzyme hydrolyzes various oxidized nucleotides, such as 8-hydroxy-dGTP and 2-hydroxy-dATP, to the corresponding nucleotide monophosphates, and acts as a nucleotide sanitization enzyme to prevent the incorporation of these modified nucleotides into DNA [7,8].

2. Materials and methods

2.1. Materials

8-OH-dGTP was prepared by the oxidation of dGTP (Sigma), and was purified by reverse-phase HPLC as described [8].

2.2. Production and purification of 8-Cl-dGTP

dGTP (5 mM; 400 μl) was reacted with 5 mM NaOCl in 250 mM sodium phosphate buffer (pH 7.4) containing 125 μM nicotine, a catalyst of chlorination caused by HOCl, at room temperature for 2 min. The reaction was stopped by the addition of 10 μl of 500 mM *N*-acetylcysteine. The 8-Cl-dGTP in the reaction mixture was isolated by preparative reverse phase HPLC using the following sequential separation steps. At first, the 8-Cl-dGTP was isolated using an eluent of 20 mM sodium phosphate buffer (pH 7.0) containing 0.6% acetonitrile (isocratic). Then, the 8-Cl-dGTP was purified from the above HPLC fraction using an eluent of 50 mM triethylammonium acetate buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 10% for 20 min in a linear gradient mode. Further purification was done using an eluent of 20 mM triethylammonium bicarbonate buffer (pH 7.0) containing 4% acetonitrile (isocratic). Finally, the isolated 8-Cl-dGTP was repeatedly lyophilized after the addition of H_2O (three times) to remove the buffer. The purity of the 8-Cl-dGTP was evaluated by its UV spectrum, and by the profile from the reverse phase HPLC using 50 mM triethylammonium acetate buffer (pH 7.0) with a linear gradient of

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0–10% acetonitrile as the eluent. Detection was performed with a Hewlett Packard 1050 HPLC detection system. For the reverse phase HPLC, an Ultrasphere ODS column (4.6×250 mm, 5 μm particle size, Beckman, CA, USA) was used. The column temperature was 30°C, and the flow rate was1.0 ml/min. The concentration of 8-Cl-dGTP was determined by the UV absorbance and the molar extinction coefficient ($\varepsilon_{max} = 1.53 \times 10^4$, $\lambda_{max} = 261$ nm at pH 7.0).

2.3. Pyrophosphatase assay

The pyrophosphatase activities were assayed in a reaction mixture (100 μl) containing 20 mM Tris–HCl (pH 8.0), 4 mM $MgCl_2$, 40 mM NaCl, 80 μg/ml bovine serum albumin, 8 mM dithiothreitol, 10% glycerol, and various amounts of nucleotide substrates. Following a preincubation at 30°C for 2 min, the mixtures were incubated at 30°C for 10 min with various amounts of the hMTH1 protein. Reactions were terminated by adding 100 μl of 5 mM EDTA. All samples were injected into a TSK-GEL DEAE-2SW column, with an isocratic elution by 50 mM phosphate buffer (pH 7.0), 1 mM EDTA, and 20% acetonitrile at a flow rate of 1 ml/min. Detection was performed with a Hewlett Packard 1040M HPLC UV detection system. The nucleoside triphosphates and their hydrolyzed products were quantified by measuring the peak areas in the HPLC data.

3. Results and discussion

When the reaction mixtures were fractionated by anion-exchange HPLC, 8-Cl-dGTP and its hydrolyzed product, 8-Cl-dGMP, were clearly separated (Fig. 1A,B). With the standard conditions described in the figure legend, 8-Cl-dGTP was hydrolyzed to 8-Cl-dGMP with 70% efficiency as compared to the 8-OH-dGTP hydrolysis, and very little dGTP degradation was found with these conditions (Fig. 1B–D). The hydrolysis rates of the three nucleotides, 8-OH-dGTP, 8-Cl-dGTP, and dGTP, with various concentrations of hMTH1, are shown in Fig. 2. The 8-Cl-dGTP was hydrolyzed at 60–80% of the rate of 8-OH-dGTP. The hydrolyzing activity of dGTP was 1.5% as compared with that of 8-OH-dGTP. Next, various amounts (1–20 μM) of 8-Cl-dGTP and 8-OH-dGTP were mixed with 1 nM hMTH1 protein, and the reaction rates were measured by anion-exchange HPLC to determine the kinetic parameters

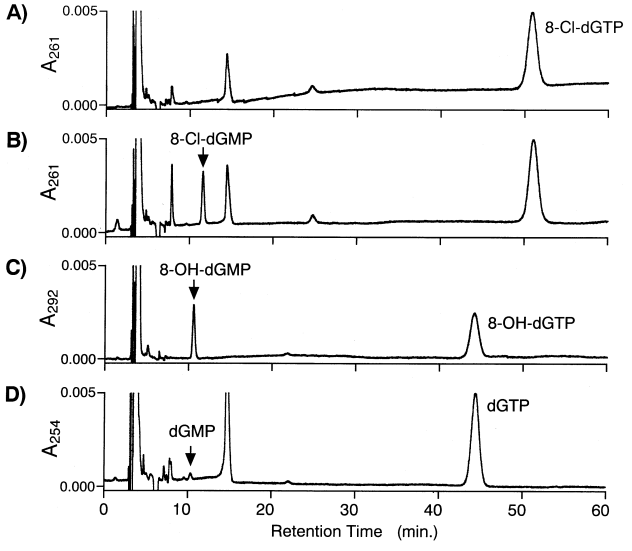

Fig. 1. Hydrolysis of oxidized nucleotides by hMTH1, monitored by anion-exchange HPLC. A, B: 5 μM 8-Cl-dGTP incubated with hMTH1 at 30°C for 0 and 5 min, respectively. C, D: 5 μM 8-OH-dGTP and dGTP, respectively, incubated with hMTH1 at 30°C for 5 min.

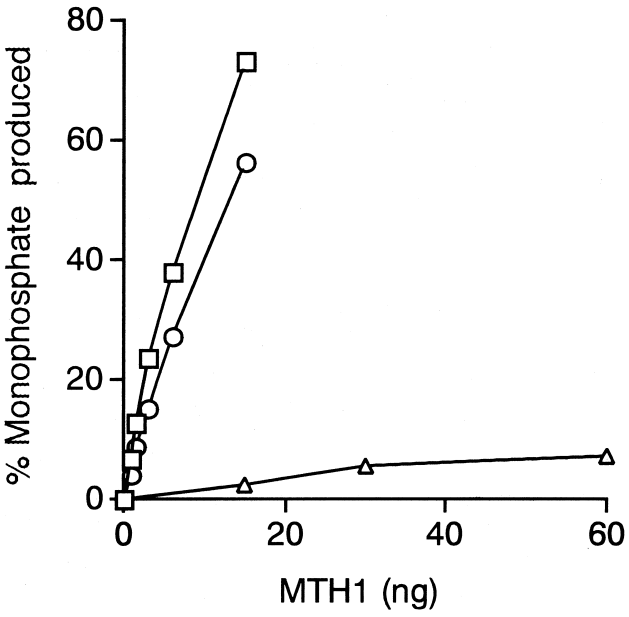

Fig. 2. Hydrolysis of 8-Cl-dGTP by hMTH1. Nucleotide substrates (5 μM) were incubated with various amounts of hMTH1 at 30°C for 5 min. The data for 8-Cl-dGTP (circles), 8-OH-dGTP (squares), and dGTP (triangles) are shown.

(Fig. 3). The kinetic efficiency (k_{cat}/K_m) of hMTH1 for 8-Cl-dGTP was half of that for 8-OH-dGTP (Table 1).

8-OH-dGDP is a strong, and competitive inhibitor of the hMTH1 activity [7]. The inhibition constants (K_i) of 8-OH-dGDP are 0.72 μM against the 2-OH-dATPase activity, and 0.51 μM against the 8-OH-dGTPase activity. The inhibitory effect of 8-OH-dGDP against the 8-Cl-dGTP activity of hMTH1 was studied. Various amounts of 8-OH-dGDP (0–0.5 μM) were added to the reaction mixtures containing 1–10 μM of 8-Cl-dGTP, and were incubated with 1 nM of hMTH1. The inhibition constant (K_i) of 8-OH-dGDP against the 8-Cl-dGTPase activity of hMTH1 was 0.43 μM. In addition, 8-OH-dGTP (5 μM) and 8-Cl-dGTP (5 μM) were mixed, and then incubated with 2 nM of hMTH1. The 8-OH-dGTPase activity and the 8-Cl-dGTPase activity were reduced to 92.4% and 76.3%, respectively, as compared with the activities for each damaged nucleotide alone. These results are in agreement with our finding that 8-Cl-dGTP is recognized with lower affinity than 8-OH-dGTP, and indicate that the damaged bases of these nucleotides share the same recognition site of hMTH1.

We have previously reported that the oxidized purine nucleotides, 2-OH-dATP, 8-OH-dATP, and 8-OH-dGTP, and 2-OH-ATP, which have the *syn*-conformation, are all good substrates for hMTH1 [7,9]. Therefore, our present result that 8-Cl-dGTP, which may adopt the *syn*-conformation

Table 1
Substrate specificity of hMTH1

Substrate	K_m (μM)	K_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
8-Cl-dGTP	58.5	19.0	0.33
8-OH-dGTP	18.9	12.4	0.66

The reaction mixtures, containing various substrates (1–20 μM of 8-Cl-dGTP or 8-OH-dGTP), were incubated with 1 nM hMTH1 for 5 min at 30°C. The k_{cat} value was calculated as molecules of product formed/molecule of hMTH1/s.

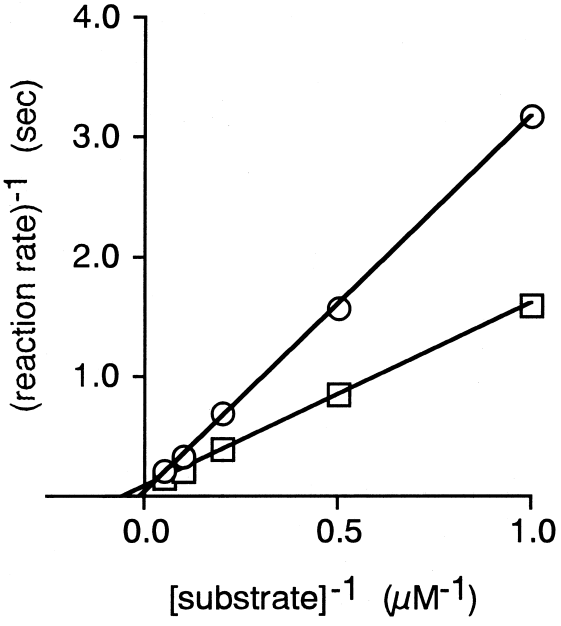

Fig. 3. Lineweaver–Burk plots for the hMTH1 activities. The data for 8-Cl-dGTP (circles) and 8-OH-dGTP (squares) were obtained from the HPLC assay, as described in Section 2. Curves were fitted to the Michaelis–Menten equation.

[10], is efficiently hydrolyzed by hMTH1 is compatible with this finding. Bessman et al. have reported that 8-Br-dATP and 8-Br-dGTP are hydrolyzed by the *Escherichia coli* Mut T protein at a higher rate than unmodified dATP and dGTP [11]. Our preliminary experiments showed that 8-Cl-dGTP may be a poor substrate for *E. coli* MutT (data not shown). This is reasonable, because MPO-dependent hypochloric acid formation is specific to the mammalian system and is not expected to occur in bacterial systems.

Our present result that 8-Cl-dGTP is a good substrate of the hMTH1 protein suggests that 8-Cl-dGTP may be produced in human cells. The detection of 8-Cl-Gua in cellular DNA and in the nucleotide pool and its mutagenic effects and repair mechanisms are important projects to be pursued in the future.

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