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Report

Thiol Reduction of 3′-Azidothymidine to 3′-Aminothymidine: Kinetics and Biomedical Implications

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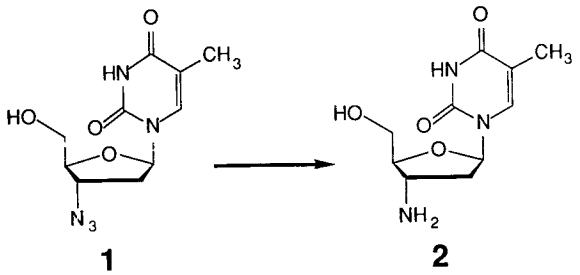
The ability of thiols to reduce 3′-azidothymidine (AZT) to 3′-aminothymidine has been investigated. Incubation with glutathione, dithiothreitol (DTT), or mercaptoethanol at pH 7.2 and 37°C leads to quantitative reduction of the azido moiety to an amine. The reaction is first order in AZT and first order in reducing agent (mono- or dithiol). The second-order rate constants are 2.77×10^{-3}, 6.55×10^{-5}, and 6.35×10^{-6} M^{-1} sec^{-1} for the dithiothreitol, glutathione, and mercaptoethanol reductions, respectively. The thiol reduction of alkyl azide to amine under mild conditions is a synthetic method particularly suitable for water-soluble azido compounds that are sensitive to catalytic hydrogenation. The potential for the mono- or dithiol-mediated reduction of alkyl azides under biological conditions must be considered when conducting studies of azido drugs.

KEY WORDS: Azide; 3′-azidothymidine (AZT); dithiothreitol (DTT); thiol.

INTRODUCTION

The success of 3′-azidothymidine (AZT; **1**) (Scheme I) as an antiretroviral drug has stimulated considerable interest in the biological activity and the chemical reactivity of azido nucleosides. Alkyl azides are readily converted to their corresponding amine by either reducing agents or catalytic hydrogenation. Thiols in particular have been shown to reduce alkyl azides under nonphysiological conditions (1) and aryl azides under physiological conditions (2). We have conducted experiments to assess the ability of thiols to reduce a model alkyl azide, AZT, under biologically relevant conditions. This paper reports investigations into the kinetics of the reduction of AZT by several common thiols including dithiothreitol (DTT), mercaptoethanol, and the cellular thiol, glutathione. AZT has been selected because it is a well-characterized azido nucleoside and because of the important implications for its activity that would be associated with any *in vivo* or *in vitro* reduction by biological thiols.



Scheme I

MATERIALS AND METHODS

AZT was generously provided by Burroughs–Wellcome. Glutathione, mercaptoethanol, and (DL)-dithiothreitol were obtained from Sigma Chemical Company. The ^{1}H NMR spectra were obtained on a General Electric GN-500 NMR spectrometer operating at 500 MHz. Mass spectral analysis was conducted on a Kratos MS-50S spectrometer.

3′-Aminothymidine (**2**). A mixture of AZT (**1**) (5.0 mg, 18.7 μmol) and DTT (14.4 mg, 93.5 μmol) in 0.1 *M* phosphate buffer (0.5 ml, pH 7.2) was stirred at room temperature, and the reaction monitored by thin layer chromatography (ethyl acetate:acetic acid, 10:1) for 1 hr, at which time no detectable AZT was left. The reaction mixture was adjusted to pH 3 with 10% HCl and applied to a cation-exchange column [Bio-Rad AG 50W-X8(H^+), 0.6 × 7 cm]. The resin was washed with water (10 ml) to remove both oxidized and reduced DTT, followed by 1 *N* NH_4OH (20 ml) to elute the product. Lyophilization gave white crystals (4.6 mg, 100% yield). The liquid secondary ion mass spectrum (LSIMS) gave (M + 1) = 242. The ^{1}H NMR spectrum (Me_2SO-d_6) was identical to the values reported in the literature (11).

Kinetic Measurements. The kinetics of AZT reduction were studied separately for each of the reducing agents. Stock solutions of AZT, DTT, glutathione, mercaptoethanol, and thymidine were prepared in 0.1 *M* potassium phosphate buffer (pH 7.2) and were degassed prior to mixing. Reaction mixtures (total volume of 0.35 ml) were 4 m*M* in AZT and 4 m*M* in thymidine. For each kinetic experiment the concentration of thiol was varied as shown in Figs. 1 and 2.

The incubation of AZT with thiols was conducted anaerobically in 0.5-ml Wheaton vials at 37°C. Aliquots (4 μl) were removed from the reaction mixtures at various time points, applied to a reverse-phase high-performance liquid chromatographic (HPLC) column (Whatman Partisil ODS-3,

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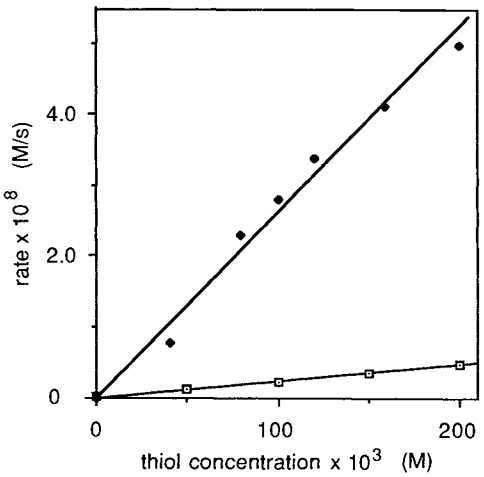

Fig. 1. Plot of the initial rate of AZT reduction versus the thiol concentration for glutathione (◆) and mercaptoethanol (□) at 37°C and pH 7.2.

0.46 × 25 cm), and eluted with 0.01 *M* potassium phosphate (pH 3.0)/methanol (75:25, v/v) (12). The absorbance was measured at 260 nm on a Kratos SF 769 variable-wavelength detector interfaced to a 3390A Hewlett–Packard reporting integrator. Thymidine was added to the reaction mixtures as an internal standard and the AZT and 3′-amino-thymidine peak areas were normalized to its area. The retention times were 3.4 min (3′-aminothymidine), 4.7 min (thymidine), and 10.8 min (AZT) at a flow rate of 1 ml/min. The initial rates were determined by plotting the percentage AZT remaining versus time.

RESULTS

Incubation of the alkyl azide, AZT, with dithiothreitol (DTT) at pH 7.2 leads to the rapid disappearance of AZT. For example, a fivefold molar excess of DTT results in the quantitative conversion of AZT to a single product in less than an hour at 25°C, as monitored by HPLC. This new compound has been isolated by ion-exchange chromatography and identified as 3′-aminothymidine based on mass spectrometry and ^{1}H NMR spectroscopy. The kinetics of reduction have been followed by monitoring the decrease in AZT and the increase in 3′-aminothymidine over time using reverse-phase HPLC as outlined under Materials and Methods. Under conditions of excess thiol the plots of ln(% AZT remaining) versus time show pseudo-first-order kinetics in AZT over two to three half-lives.

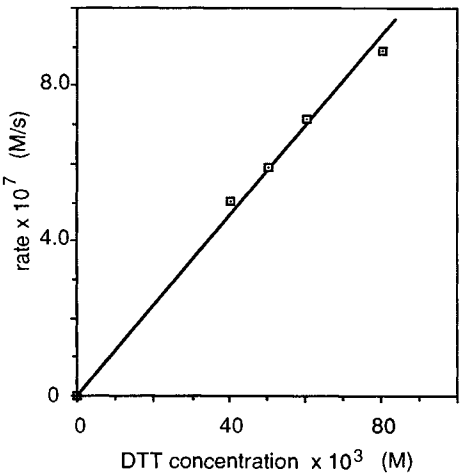

Fig. 2. Plot of the initial rate of AZT reduction versus dithiothreitol concentration at 37°C and pH 7.2.

Table I. Second-Order Rate Constants × 10^5 (M^{-1} sec^{-1})

DTT	277
Glutathione	6.55
Mercaptoethanol	0.635

The dependence of the rate of AZT conversion on the concentration of thiol has been studied with three common reducing agents, DTT, mercaptoethanol, and glutathione. As can be seen in Figs. 1 and 2, the plots of initial rate versus thiol concentration are first order in thiol reagent. The rate constants were calculated from the slopes of these plots according to k = slope/$[AZT]_0$ and are given in Table 1.

DISCUSSION

The susceptibility of alkyl and aryl azides to reduction by thiols has been largely ignored in biochemical studies involving azide containing compounds. Knowles *et al.* (2) have pointed out that DTT will cause the rapid reduction of aryl azide photoaffinity reagents, thus rendering them impotent. On the other hand, the initial investigation of the ability of AZT to inhibit human immunodeficiency virus replication involved incubating virus-exposed cells in a medium containing AZT and a 50-fold molar excess of mercaptoethanol for 10 days (3).[3] The results in Table 1 demonstrate that the consequences of thiol-mediated reduction need to be considered in both *in vitro* and *in vivo* studies. The presence of up to 20 m*M* reduced glutathione in the cytoplasm of most cells means that the potential exists for the intracellular conversion of azide-containing compounds to amines. Although DTT is not a naturally occurring thiol reductant, there are sources of dithiols inside the cell, e.g., lipoamide and the active sites of enzymes including ribonucleotide reductase, lipoamide dehydrogenase (diaphorase), and glutathione reductase. Despite the strong implications for AZT metabolism that these results provide, it must be emphasized that aminothymidine has not been detected as a metabolite in any *in vivo* or *in vitro* studies so far. Thus, the significance of thiol-mediated AZT reduction toward AIDS chemotherapy is yet to be determined.

Cellular reduction of alkyl azides by thiols represents a new avenue for the design of prodrugs of amine-containing

[3] Under the conditions of that study (3) a negligible amount of AZT would be reduced; however, replacement of mercaptoethanol by DTT would convert ca. 10% of the AZT to aminothymidine.

compounds (4,5).[4] Such a strategy could take advantage of the enhanced lipophilicity of azido compounds and their ability to pass readily through cell membranes by nonfacilitated diffusion (6). Once inside the cell reduced glutathione, or other thiols, could then effect the conversion to the corresponding biologically active amine. This approach might be particularly appropriate for delivering drugs to the strongly reducing environment found in hypoxic tissue.

Finally, the reduction of alkyl azides by DTT has significant synthetic applications as well. Generally, hydride reducing agents or catalytic hydrogenation is used for the preparation of primary amines from their corresponding azides. However, these same reagents also reduce a wide range of other functional groups. More selective reagents for the reduction of azides in organic solvents have been explored including propane-1,3-dithiol/triethylamine (1), triphenylphosphine/ammonium hydroxide (7,8), and stannous chloride (9). Of the three thiol reducing agents studied in this report, DTT is the most rapid. DTT affords a *quantitative* reduction of alkyl azide in aqueous solution at neutral pH and room temperature. In contrast, the yields using the standard catalytic hydrogenation methods for the reduction of AZT to 3′-aminothymidine are reported to range from only 57% (10) to 67% (11). Moreover, DTT is ideally suited for the reduction of azido compounds containing functional groups vulnerable to catalytic hydrogenation, e.g., in the synthesis of amino sugars and other amino nucleosides from their corresponding azides. Finally, DTT is biocompatible: it can reduce azido compounds in biological media and in the presence of enzymes or other biomolecules.

[4] For example, in the case of AZT there could be local reduction to 3′-aminothymidine. Lin *et al.* (4) report that 3′-aminothymidine has a lower antiviral activity than 3′-azidothymidine. However, 3′-aminothymidine triphosphate is as effective as AZT triphosphate (the active form of AZT) in inhibiting the reverse transcriptase of HIV, and it is less inhibitory toward the human DNA polymerase (5).

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