This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2003)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2003 Term

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Dithiothreitol (Cleland's Reagent)

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For 77:222, Spring 2003
For April 7, 2003

Abbreviations:

CoA,	coenzyme A
CoA-SS-CoA,	oxidized coenzyme A
Cu-DTT,	copper-dithiothreitol complex
DTT, DTT^{red},	1,4- dithiothreitol
DTT^{ox},	oxidized dithiothreitol
$DTT^{\bullet}$,	dithiothreitol radical
EDTA,	ethylenediaminetetraacetic acid
GSH,	glutathione
GSSG,	glutathione disulfide
HPLC,	high-performance liquid chromatography
NMR	nuclear magnetic resonance
RSH,	compounds containing thiol groups.
RSSR,	disulfide compounds
UV,	ultraviolet

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Abstract

Dithiothreitol (DTT), also known as Cleland's Reagent, contains two thiol groups and is commonly used to reduce disulfide bond in proteins. It can protect many thiol-containing biomolecules such as coenzyme A (Co-A), GSH (glutathione) from forming disulfide bonds. Dithiothreitol can not only scavenge hydroxyl radical that is readily to attack DNA and proteins, but also react with hydrogen peroxide that may generate hydroxyl radicals through the Fenton reaction. However, in the presence of metals and oxygen, dithiothreitol can generate many free radicals including hydroxyl radicals to induce oxidative damage in proteins, lipids and DNA. Dithiothreitol is also a strong chelating reagent that can form specific and very stable polymeric and monomeric complexes with metal ions using its two thiol groups. HPLC (high-performance liquid chromatography) and UV (ultraviolet) spectrum are the common used methods to detect both oxidized DTT (DTT^{ox}) and DTT.

Introduction

1,4-Dithiothreitol (DTT) is also known as Cleland's Reagent. Its chemical formula is $C_4H_{10}O_2S_2$ shown in Figure 1. It is water-soluble and can readily permeate cell membrane. It contains two thiol groups and two hydroxyl groups. So it is commonly used in biochemical research to protect sulfhydryl groups from oxidation or reduce disulfide linkages to free sulfhydryl groups in proteins and enzymes. Since the reversible formation of disulfide bonds is involved in many biological processes including protein activation and inactivation, regulation of gene expression, dithiothreitol may affect many biological processes by regulating protein activity. This mini review will focus on its reactions and its protective functions for thiol groups.

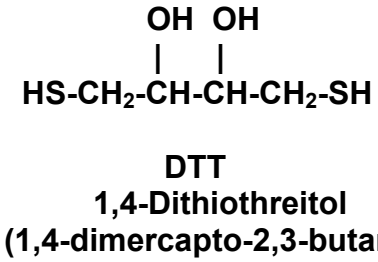


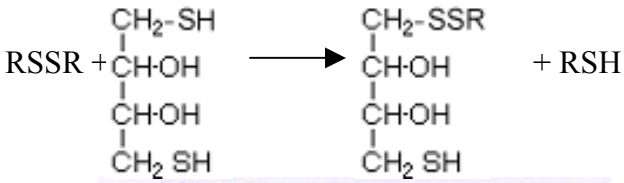
Figure 1: Chemical structure of dithiothreitol, which is also called 1,4-dithiothreitol, threitol and 1,4-dimercapto-2, 3-butanediol.

Physical characteristics

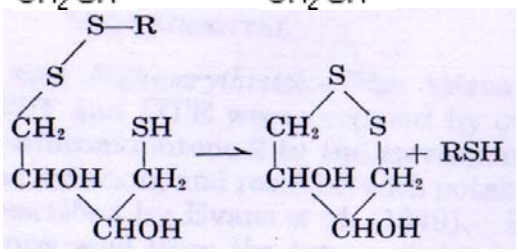
Dithiothreitol has the molecular weight of 154.26 Da. It appears as white powder or needles. It is soluble in water, ethanol, acetone, and ethyl acetate. It has thiol odor, and its boiling point and melting point are125-130^{o}C (257-266F) and 42 – 43^{o}C (108 - 109F) respectively [1]. Due to its low redox potential (-0.332 V at pH 7 and –0.366 V at pH 8.1), DTT can reduce disulfides very rapidly and maintain thiol groups with high efficiency [2]. The ideal storage temperature is +2^{o}C - +8^{o}C. DTT has little tendency to be oxidized directly by air, so it has advantages to be a protective reagent compared to other thiol compounds.

Dithiothreitol as a protective reducing reagent

Thiol groups are susceptible to be oxidized and generate disulfides, such as coenzyme A and glutathione. To maintain these thiol groups, another thiol group as a sacrificial molecule with a lower redox potential will exchange the thiol group with the oxidized thiol group. This is a major reaction for DTT to protect thiol groups (reactions 1 and 2) [2]. Reaction 3 is the final reaction of reaction 1 and 2. Two thiol groups in DTT have the first and second p*K*a values of 9.2 and 10.1 respectively [1].



(1)



(2)

$$RSSR + DTT^{red} \longleftrightarrow 2\ RSH + DTT^{ox} \qquad (3)$$

In these reactions, RSSR can be many disulfide compounds that are formed by thiol groups such as cysteine, and glutathione [3]. The equilibrium constant for reduction of cystine by DTT is 1.3×10^4 (reaction 1) and the equilibrium constant for the following cyclization reaction with cystine is about 1×10^4 (reaction 2) [2]. The reactions of DTT consist of the complete reduction of intra- or inter-molecular disulfide bonds *via* a thiol-disulfide interchange process. Figure 2 shows that DTT keeps coenzyme A reduced [2]. DTT can stabilize thiol-containing proteins or enzymes; at high concentrations, it may also inactivate some disulfide-containing proteins.

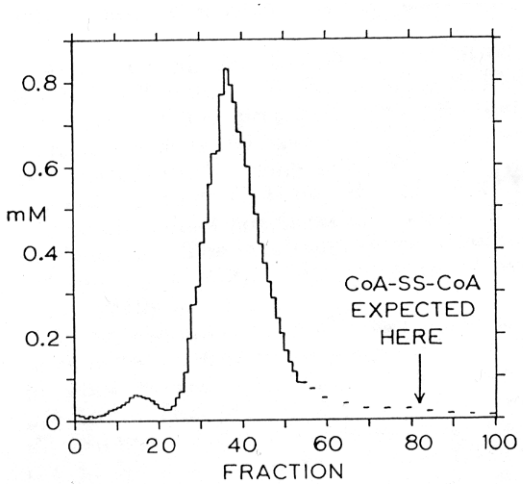
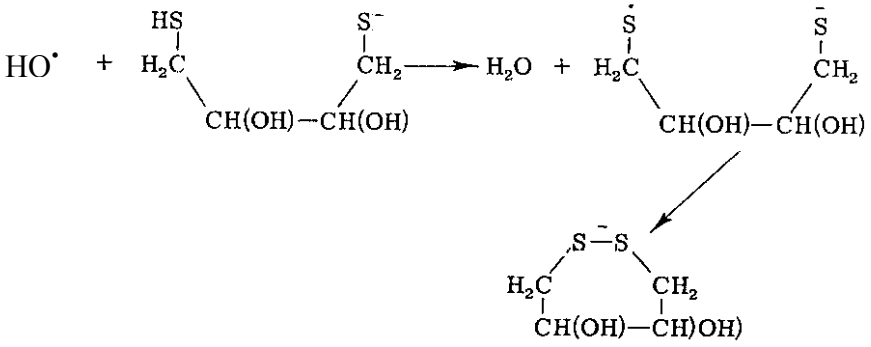


Figure 1: chromatography of coenzyme A on diethyl-aminoethyl-cellulose-bicarbonate in presence of DTT. No CoA-SS-CoA was detected in the CoA-SS-CoA expected fractions. However, chromatography of the same prepared coenzyme A in absence of DTT gives several small peaks (presumably mixed disulfides) in addition to the major peaks of reduced and oxidized CoA. Adapted from [2].

Dithiothreitol was found as a radioprotector, since many sulfydryl compounds have been shown to scavenge damaging free radicals. It was reported that dithiothreitol could react with primary water radicals and some secondary free radicals using pulse radiolysis techniques [4]. Hydroxyl radicals react with DTT with $k = 1.5 \times 10^{10}\ M^{-1}\ s^{-1}$ [4].

$$^{\bullet}OH + DTT \rightarrow Products \qquad (4)$$



(5)

RSSR$^{\bullet -}$ form of DTT

The RSSR$^{\bullet -}$ radical of DTT can react with oxygen to generate superoxide. DTT can also repair the organic free radicals that are generated by radiolysis [4].

$$RSSR^{\bullet -} + O_2 \rightarrow RSSR + O_2^{\bullet -} \qquad k = 2.8 \times 10^{9}\ M^{-1}\ s^{-1} \qquad (6)$$

$$CH_3C^{\bullet}(OH)CH3 + DTT \rightarrow CH_3CH(OH)CH_3 + DTT^{\bullet} \qquad k = 2.1 \times 10^{8}\ M^{-1}\ s^{-1} \qquad (7)$$

Dithiothreitol as a chelating reagent

Thiol groups provide biological targets for metal ions. Many toxic metals exert their deleterious effect through inhibition of thiol enzymes. DTT was studied as a target or model for biological heavy metal ion binding and reactivity [5]. DTT is a strong chelating reagent. DTT can form polymeric complexes with many heavy metals, examples are shown in Figure 2 [5].

a)

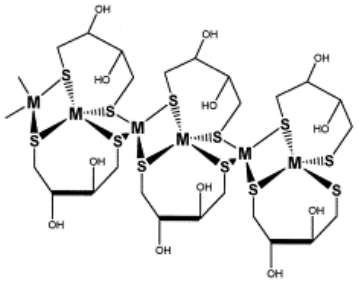


Figure 2a: Schematic representation of structures of polymeric complexes of divalent tetrahedral metal ions and DTT. M represents Zn (II), Cd(II), Pb(II).

b)

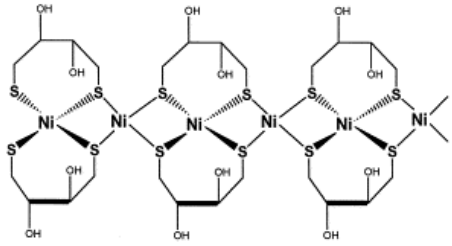


Figure 2b: Schematic representation of structures of polymeric complexes of square planar Ni(II) and DTT. (Adapted from [5])

Toxicity induced by dithiothreitol

At certain concentrations (less than about 0.1 mM or greater than about 1 mM), the thiol-containing compounds are generally good antioxidants and protect thiol groups from oxidation [6,7], but they decrease the clonogenicity at intermediate concentrations [6]. In the presence of metals and oxygen, DTT may induce free radicals and cause damage in proteins, lipids and DNA [6]. During oxidation of DTT, hydroxyl radicals are generated and induce cell killing and apoptosis [7]. Fe^{3+} catalyzes the oxidation of DTT and is reduced to Fe^{2+} (reaction 8). This reaction is the rate-limiting step and may initiate a free radical chain of reactions [6]. EDTA (ethylenediaminetetraacetic acid) stimulates iron-catalyzed oxidation of DTT probably by

accelerating Fe^{3+} reduction [6]. There are two mechanisms to generate H_2O_2 and DTT radicals following iron-catalyzed DTT oxidation (reaction 8, 9,10, 11, 12) [6]. The rate constant of reaction 8 is about 0.7–0.8 M^{-1} s^{-1} in the absence of EDTA and about 2.3–2.8 M^{-1} s^{-1} in the presence of EDTA. Both mechanisms II and I contribute to the generation of H_2O_2 and DTT radical ($RS^{\bullet}$). Mechanism II may be more important than mechanism I, since the rate constant of reaction 12 is k =10^7 M^{-1} s^{-1}, which is greater than that for the spontaneous dismutation of superoxide to form H_2O_2 *via* reaction 10 (k = 8 x10^4 M^{-1} s^{-1}) [6].

$$2RSH + 2Fe^{3+} \rightarrow 2RS^{\bullet} + 2Fe^{2+} + 2H^{+} \quad (8)$$

Mechanism 1:

$$2Fe^{2+} + 2O_2 \rightarrow 2O_2^{\bullet -} + 2Fe^{3+} \quad (9)$$

$$2O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (10)$$

Sum:

$$2RSH + O_2 \rightarrow H_2O_2 + 2RS^{\bullet} \quad (11)$$

Mechanism 2:

$$Fe^{2+} + O_2 \rightarrow O_2^{\bullet -} + Fe^{3+} \quad (9)$$

$$Fe^{2+} + O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + Fe^{3+} \quad (12)$$

Sum:

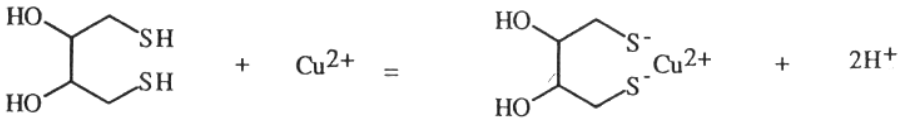
$$2RSH + O_2 \rightarrow H_2O_2 + 2RS^{\bullet} \quad (11)$$

During these processes, many free radicals are generated. Fenton reaction can also be carried out to generate hydroxyl radicals that are very reactive to induce damage.

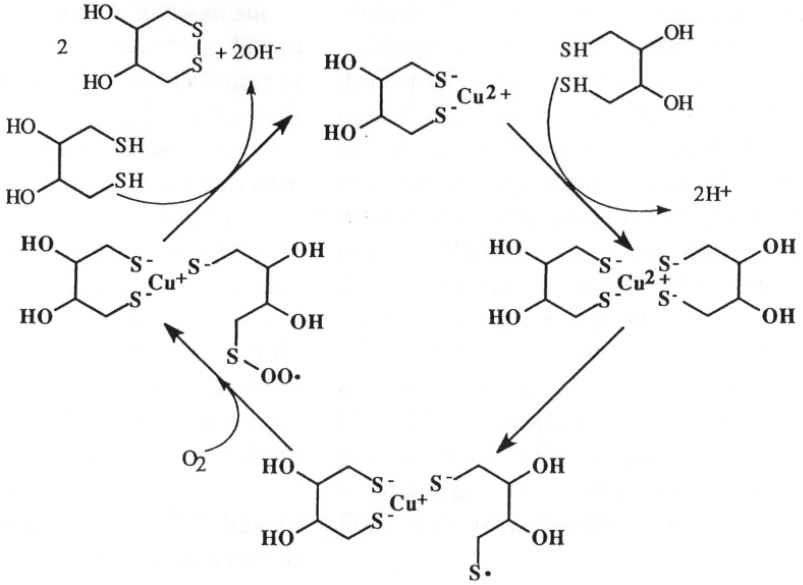
In addition to iron, copper can catalyze the oxidation of DTT (Figure 8). Copper-DTT complex is formed during the process (reaction 13). Cu-DTT complex catalyzes DTT oxidation (reaction 14). In reaction 14, the coordinating Cu^{2+}, which is [Cu^{2+} (DTT^{2-})], is able to chelate a second DTT molecule to form [Cu^{2+} (DTT^{2-})$_2$]$^{2-}$ (reaction ①), then an intramolecule electron is transferred from sulfur to copper and [Cu^{+} (DTT^{2-})($DTT^{\bullet -}$)]$^{2-}$ is generated (reaction ②). The thiyl sulfur atom in $DTT^{\bullet -}$ has lost its negative charge and is released from binding with central copper ion. Thiyl sulfur atom reacts with oxygen and produces sulfur peroxide (reaction ③). DTT is a

reductant for the sulfur peroxide and Cu^{2+}-DTT complex is reproduced (reaction ④). Superoxide (reaction 15) and H_2O_2 (reaction 16) can be produced in the absence of free DTT, and hydroxyl radicals are generated *via* reduction of H_2O_2 by cuprous ion (reaction17) [7].

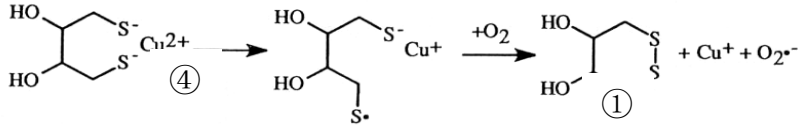
Generation of Cu-DTT complex



2. Cu-DTT catalyzed DTT oxidation (13)



Oxidation of Cu-DTT in the absence of free DTT



(15)

$$Cu^{+} + O_2^{\bullet -} + 2H^{+} \rightarrow Cu^{2+} + H_2O_2$$

③ ②

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^{-} \quad (17)$$

So DTT generates many free radicals that can damage proteins, DNA, and lipids. The oxidation of DTT may induce great toxicity when exposed to oxygen and metals.

Detection of dithiothreitol

There are many methods to detect DTT and its oxidized form. Ultraviolet (UV)-visible spectrum [2,5], HPLC (high-performance liquid chromatography) [5, 8] and NMR (nuclear magnetic resonance) [5] are the most popular methods. DTT and its oxidized form have different ultraviolet spectrums [2]; Oxidized DTT has an absorption peak at the wavelength about 283 nm, while DTT has great absorption at relatively short wavelength about 200 nm [2, 6]. The amount of the oxidized forms present can be determined from their ultraviolet spectrum [2]. Both DTT and its oxidized form can be determined during reversed-phase HPLC analysis by their absorption at 210 nm (Figure 3)[3, 6,9]. Prontonation constants of DTT and stability constants of its metal complex can be measured by using pH-metric titration [5].

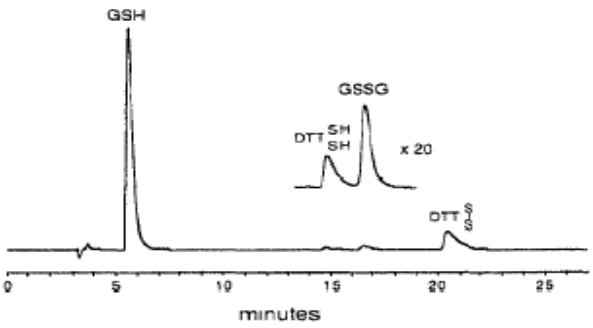


Figure 3: HPLC chromatogram of an equilibrium mixture of GSH, GSSG, DTT^{ox}, DTT^{red}. HPLC can separate each opponent, and concentrations can be measured by HPLC peaks. The separated DTT^{ox} and DTT^{red} can be further determined by their UV absorption at 210nm. (Adapted from [9]).

Summary

Dithiothreitol is a well-known reducing reagent, which can protect thiol groups from oxidation. Due to its low redox potential, it can also cut disulfide bonds and reduce the disulfide compounds. Recently, it was founded that dithiothreitol is a strong chelating reagent which can

form complexes with many heavy metals. So the effect of dithiothreitol should be taken as a major problem if the binding of heavy metal to thiol-containing proteins or peptides is to be studied. Moreover, metal-catalyzed dithiothreitol oxidation generates many damaging free radicals such as hydroxyl radical and superoxide that directly inactivate many proteins and DNA. So dithiothreitol is not always a protective antioxidant, it also can induce oxidative damage to biomolecules.

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