ORGANIC LETTERS

2004
Vol. 6, No. 21
3809−3812

5-(2-Aminoethyl)dithio-2-nitrobenzoate as a More Base-Stable Alternative to Ellman's Reagent

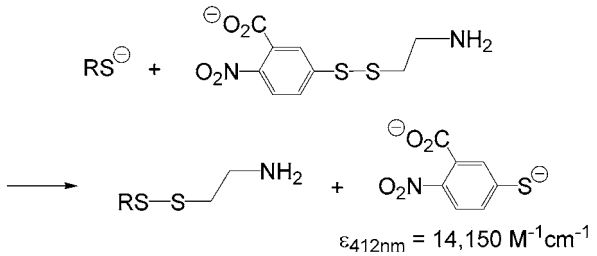
Jinge Zhu, Ilirian Dhimitruka, and Dehua Pei*

Department of Chemistry and Ohio State Biochemistry Program, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

pei.3@osu.edu

Received August 11, 2004

ABSTRACT



5-(2-Aminoethyl)dithio-2-nitrobenzoate (ADNB) reacts with free thiols with kinetics similar to those of Ellman's reagent but has dramatically improved stability under alkaline conditions, making it an excellent alternative to Ellman's reagent for the quantitation of thiol contents and enzymatic assays under basic pH conditions.

Since its introduction in 1959, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) has been the most popular reagent for the quantitation of sulfhydryl contents as well as covalent modification of thiol groups on proteins.[1] DTNB reacts rapidly and completely with free thiols or accessible protein thiols to release an equivalent of a highly chromogenic product, 5-thio-2-nitrobenzoate (TNB) (ϵ = 14 150 M^{-1} cm^{-1} at 412 nm).[1,2] This property has also rendered DTNB very useful for the kinetic assay of a wide variety of enzymes such as acetylcholine esterase,[3] carboxypeptidase A,[4] phospholipase A2,[5] peptide deformylase,[6] methionine aminopeptidase,[7] arginase,[8] cystathionine β-synthase,[9] *S*-ribosylhomocysteinase (LuxS),[10] and *S*-adenosylmethionine-dependent methyltransferases.[11] Typically, enzymatic action on the natural or an artificial substrate results in the release of a free thiol, which is quantitated by reacting with DTNB and measuring absorbance at 412 nm. Despite its many advantages, DTNB suffers from a major drawback as a thiol-quantifying reagent, i.e., its extreme sensitivity to pH. At pH < 6, the reaction between DTNB and free thiols is slow and often becomes rate limiting. At pH > 9, DTNB undergoes hydrolytic cleavage of the activated disulfide, resulting in high background signals.[12] The problem of sluggish reaction at low pH has largely been resolved by replacing DTNB with 4,4′-dipyridyl disulfide, whose reactiv-

(1) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.
(2) Eyer, P.; Worek, F.; Kiderlen, D.; Sinko, G.; Stuglin, A.; Simeon-Rudolf, V.; Reiner, E. *Anal. Biochem.* **2003**, *312*, 224−227.
(3) Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Feather-Stone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88−95.
(4) Brown, K. S.; Kingsbury, W. D.; Hall, N. M.; Dunn, G. L.; Gilvarg, C. *Anal. Biochem.* **1987**, *161*, 219−225.
(5) Yu, L. Ternansky, R. J.; Crisologo, J. F.; Chang, J.; Baker, B. L.; Coutts, S. M. *Anal. Biochem.* **1998**, *265*, 35−41.
(6) Guo, X.-C.; Rajagopalan, P. T. R.; Pei, D. *Anal. Biochem.* **1999**, *273*, 298−304.
(7) Zhou, Y.; Guo, X.-C.; Yi, T.; Yoshimoto, T.; Pei, D. *Anal. Biochem.* **2000**, *280*, 159−165.
(8) Han, S.; Viola, R. E. *Anal. Biochem.* **2001**, *295*, 117−119.
(9) Aitken, S. M.; Kirsch, J. F. *Biochemistry* **2003**, *42*, 571−578.
(10) Zhu, J.; Dizin, E.; Hu, X.; Wavreille, A.-S.; Park, J.; Pei, D. *Biochemistry* **2003**, *42*, 4717−4726.
(11) Hendricks, C. L.; Ross, J. R.; Pichersky, E.; Noel, J. P.; Zhou, Z. S. *Anal. Biochem.* **2004**, *326*, 100−105.
(12) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49−60.

10.1021/ol048404+ CCC: $27.50
Published on Web 09/22/2004

ity is enhanced by protonation.[13] On the other hand, the high background signal associated with its alkaline hydrolysis has not yet been effectively addressed.

The susceptibility of DTNB to alkaline hydrolysis is due to the presence of two powerfully electron-withdrawing nitro groups, which render the aryl disulfide highly electrophilic (activated disulfide). Aliphatic disulfides (R−S−S−R) and aryl disulfides (Ar−S−S−Ar) that do not bear electron-withdrawing groups are quite stable to alkaline conditions. We reasoned that an asymmetric disulfide formed between an aliphatic thiol and TNB should have reduced reactivity toward hydroxide. A number of asymmetric disulfides of this type have previously been developed to quantitate proteinogenic thiols that reside in anionic or hydrophobic environments and thus fail to react or react very slowly with the doubly negatively charged DTNB.[14−16] Thiols attack exclusively on the aliphatic sulfur to release TNB. It was reported that at neutral pH the asymmetric disulfides react rapidly with free thiols, with reaction rates only 2−5-fold slower than that of DTNB.[14] One report noted that the background hydrolysis of an asymmetric disulfide was 13-fold slower than DTNB at pH 8.5.[16] Surprisingly, to the best of our knowledge, a complete evaluation of the reactivity of asymmetric disulfides toward free thiols and hydroxide has not been carried out. In this work, we determined the reaction kinetics of an asymmetric disulfide, 5-(2-aminoethyl)-dithio-2-nitrobenzoate (ADNB), toward free thiols and background hydrolysis over the pH range of 5−13. Our results demonstrate ADNB as an excellent thiol-quantifying reagent with much improved stability under alkaline conditions.

ADNB was chosen because of its ease of preparation, which involves reacting 2-aminoethanethiol with DTNB in an aqueous solution (pH 7).[14,17] The zwitterionic ADNB precipitated out of the solution and was readily isolated by filtration. Aqueous stock solutions of ADNB were prepared by dissolving the solid in 1 M HCl and diluted to the desired concentrations in water.

DTNB and ADNB were first compared for their background hydrolysis rates over the pH range of 5−13. At pH ≤ 8, both DTNB and ADNB are quite stable, showing similar and very slow background hydrolysis (pseudo-first-order rate constant $k_{OH} < 1 \times 10^{-4}$ s^{-1}) (Figure 1 and Table 1). As the pH increases, the hydrolysis rates of both DTNB and ADNB increase. However, the magnitude of rate increase with pH is greater for DTNB than ADNB (Figure 1b). At pH 10, DTNB was hydrolyzed 14-fold faster than ADNB [$k_{OH} = 1.8 \times 10^{-3}$ s^{-1} (DTNB) vs 1.3×10^{-4} s^{-1} (ADNB)] (Figure 1a). At pH 13, DTNB was rapidly hydrolyzed at a pseudo-first-order rate of 0.2 s^{-1}, whereas the hydrolysis of ADNB was 41-fold slower ($k_{OH} = 4.9 \times 10^{-3}$ s^{-1}) (Figure 1b and Table 1). Note that for either DTNB or ADNB, the hydrolysis rates (k_{OH} values) did not increase linearly with hydroxide concentration (<10-fold for every unit of pH increase). This is likely due to the different reaction mechanisms operative under different pH conditions; when the hydroxide concentration is low, the initial product, 3-carboxylate-4-nitrobenzenesulfenate ion (Ar−SO$^-$), can react with another molecule of DTNB (or ADNB) to release a second equivalent of TNB, whereas at high hydroxide concentration Ar−SO$^-$ reacts with the hydroxide ion instead.[12]

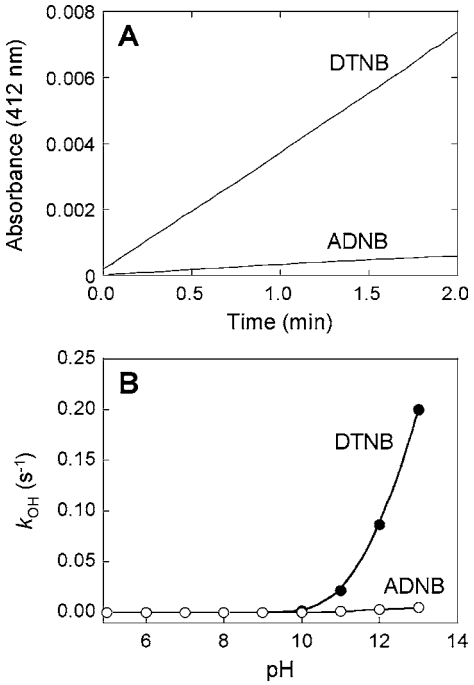


Figure 1. Comparison of background hydrolysis rates of DTNB and ADNB: (A) hydrolysis reaction progress curves of DTNB and ADNB at pH 10; (B) dependence of DTNB and ADNB hydrolysis rates on pH.

Table 1. Reactivity of DTNB and ADNB toward β-Mercaptoethanol and Hydroxide[a]

	DTNB		ADNB	
pH	k_{RSH} ($M^{-1}s^{-1}$)	k_{OH} ($\times 10^{-6}$ s^{-1})	k_{RSH} ($M^{-1}s^{-1}$)	k_{OH} ($\times 10^{-6}$ s^{-1})
5	66	9.4	21	4.7
6	115	19	69	19
7	219	33	153	28
8	1600	71	1200	66
9	21 000	410	17 000	110
10	35 000	1800	28 000	130
11	75 000	22 000	59 000	1200
12	81 000	87 000	64 000	3100
13	94 000	200 000	67 000	4900

[a] Each value shown represents the mean from seven repetitions of the experiment. Key: k_{RSH}, rate constant of reaction with β-mercaptoethanol; k_{OH}, rate constant of alkaline hydrolysis.

(13) Le, M.; Means, G. E. *Anal. Biochem.* **1995**, *229*, 264−271.
(14) Zhang, H.; Le, M.; Means, G. E. *Bioorg. Chem.* **1998**, *26*, 356−364.
(15) Faulstich, H.; Heintz, D. *Method Enzymol.* **1995**, *251*, 357−366.
(16) Faulstich, H.; Tews, P.; Heintz, D. *Anal. Biochem.* **1993**, *208*, 357−362.
(17) Witzemann, V.; Muchmore, D.; Raftery, M. A. *Biochemistry* **1979**, *18*, 5511−5518.

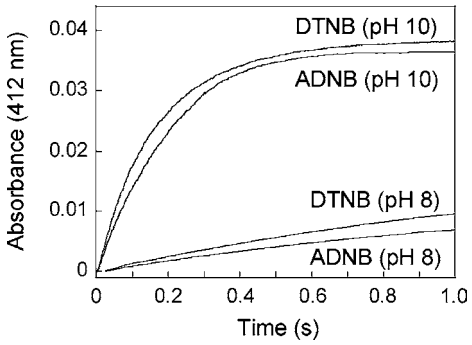
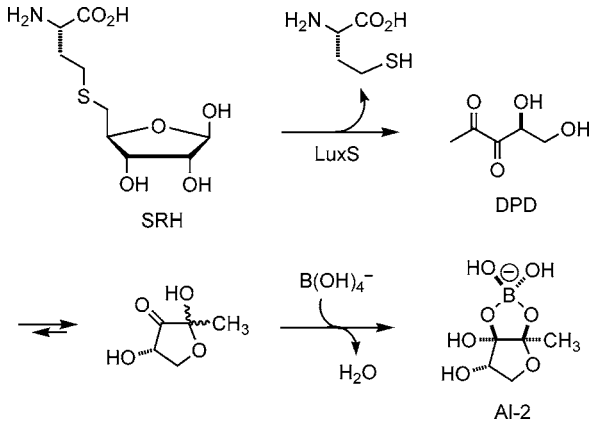


Figure 2. Reaction progress curves of DTNB and ADNB (150 μM) with β-mercaptoethanol (25 μM) at pH 8 and 10.

Next, the reactivity of DTNB and ADNB toward free thiols was compared. Both DTNB and ADNB reacted very rapidly with β-mercaptoethanol. The reactions were initiated by mixing the free thiol (25 μM) and DTNB (or ADNB) (150 μM) in buffers of various pH (pH 5−13) in a rapid-mixing apparatus. The reaction progress was monitored continuously at 412 nm (Figure 2). The second-order rate constants (k_{RSH}) were calculated from the slopes of the early regions of the progress curves. At pH ≤ 7, DTNB reacts slightly faster than ADNB (~2-fold) (Table 1), consistent with a previous report.[14] At higher pH, both DTNB and ADNB showed pH-dependent increase in reaction rates, likely as a result of increased ionization of free thiol into the more nucleophilic thiolate ion (Table 1). The plot of the k_{RSH} values in Table 1 against pH gave a pK_a of 8.5 for β-mercaptoethanol, which is lower than the reported value of 9.5, probably due to the presence of 150 mM NaCl in our assay buffers.[18] Unlike the background hydrolysis reaction, however, the reactions of β-mercaptoethanol with DTNB and ADNB show very similar pH dependence and have very similar reaction rates over the entire pH range of 8−13 (Table 1). Quantitative release of TNB from DTNB and ADNB was observed for β-mercaptoethanol as well as all other thiols tested (e.g., 2-mercaptoacetic acid, 4-mercaptobutyric acid, thiolacetic acid, cysteine, and homocysteine).

The utility of ADNB in enzymatic assays was tested by determining the pH profile of *S*-ribosylhomocysteinase (LuxS), a key enzyme involved in the biosynthesis of type 2 bacterial autoinducer (AI-2).[19] LuxS cleaves the thioether bond in *S*-ribosylhomocysteine (SRH) to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione (Scheme 1).[20] LuxS reactions were carried out in buffers of varying pH (pH 6−10.5) that contained SRH (0−70 μM) and a fixed concentration of ADNB (240 μM). The reactions were initiated by the addition of Co(II)-substituted *Escherichia coli* LuxS (0.4 μM) and continuously monitored at 412 nm. The initial rates obtained from the reaction progress curves were fitted to the Michaelis−Menten equation to obtain the

Scheme 1. Reaction Catalyzed by *S*-Ribosylhomocysteinase (LuxS) and Formation of AI-2



k_{cat} and K_M values. Interestingly, neither the k_{cat} value (~0.44 s^{-1}) nor the K_M value of LuxS (~16 μM) showed any significant pH dependence over the pH range of 6−10 (Figure 3). The observed slight decrease in activity at pH ≤ 6 or pH > 10 was due to enzyme denaturation at extreme pH conditions. To ascertain that the flat pH profile was not due to the use of ADNB, we repeated the profile in pH range of 6−10 by using DTNB to monitor the thiol release. An identical pH profile was obtained (not shown). Next, the pH profile of *Bacillus subtilis* LuxS was attempted. This had previously been a difficult task by using DTNB to monitor the thiol release because *B. subtilis* LuxS has a very slow turnover number (0.03 s^{-1})[10] and the background hydrolysis of DTNB at basic pH was problematic. However, this difficulty is easily overcome by employing ADNB in LuxS assays at pH 9−10.5 (DTNB was still used for assays at pH ≤ 8 since it reacts with thiols slightly faster than ADNB at acidic pH). Like the *E. coli* enzyme, *B. subtilis* LuxS has an essentially flat pH profile for both the k_{cat} value (0.03 s^{-1}) and its K_M value (2.4 μM) (Figure 3).

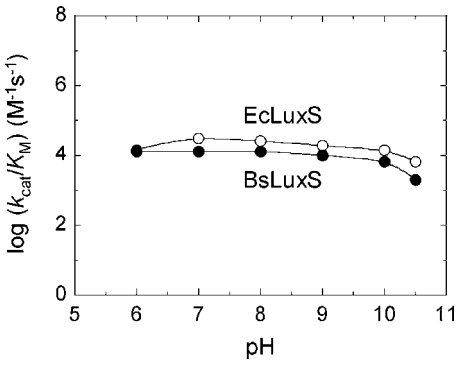


Figure 3. Effect on pH on the catalytic activity (k_{cat}/K_M value) of *E. coli* and *B. subtilis* LuxS. The buffers used were 50 mM MES (pH 6), 50 mM HEPES (pH 7−8), 50 mM CHES (pH 9), and 50 mM CAPS (pH 10 and 10.5). All buffers also contained 150 mM NaCl.

(18) Dean, R. L. *Am. Biol. Teacher* **2002**, *64*, 620−627.
(19) Surette, M. G.; Miller, M. B.; Bassler, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1639−1644.
(20) Pei, D.; Zhu, J. *Curr. Opin. Chem. Biol.* **2004**, *8*, in press.

In conclusion, ADNB reacts with free thiols with similar kinetics to DTNB but with dramatically improved stability under alkaline conditions. It thus provides a useful alternative to Ellman's reagent for the quantitation of thiol contents and enzyme kinetic assays under basic pH conditions.

Acknowledgment. We thank Marina Bakhtina for assistance in the stopped-flow reaction analyses. This work was supported by the National Institutes of Health (AI40575 and GM62820).

Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

OL048404+