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A Kinetic Approach to Characterize the Electrostatic Environments of Thiol Groups in Proteins

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In this study, we synthesized a zwitterionic DTNB derivative, 5-(2-aminoethyl)-dithio-2-nitrobenzoate (ADNB), and characterized its reactions with several cationic, anionic, and neutral thiols. Reactions with ADNB, unlike those with DTNB, are relatively insensitive to electrostatic environments and ionic strengths. At relatively low ionic strength, rate ratios, $k_{\mathrm{ADNB}}/k_{\mathrm{DTNB}}$, varied from 0.22 for reactions with low-molecular-weight cationic thiols to 3.0 for those with low-molecular-weight anionic thiols. A $k_{\mathrm{ADNB}}/k_{\mathrm{DTNB}}$ ratio of ~200 for Cys-34 of BSA appears to reflect a very anionic environment. $k_{\mathrm{ADNB}}/k_{\mathrm{DTNB}}$ ratios of ~6 and ~1, respectively, for canine and equine serum albumins, which have Glu-82 → Asp and Glu-82 → Ala substitutions suggest Glu-82 is the most important anionic residues affecting the reactivity of Cys-34 in BSA. $k_{\mathrm{ADNB}}/k_{\mathrm{DTNB}}$ ratios appear to be useful for characterizing electrostatic environments of thiol groups in proteins.

INTRODUCTION

Thiol groups are required for the catalytic activities of many enzymes, are found in ligand and receptor binding sites, and are required for the normal structure and functional properties of many proteins. They vary in reactivity due to differences in their accessibility, their proximity to charged groups and other features of their environments.

5,5′-Dithiobis(2,2′-nitrobenzoate) (DTNB)[2] is one of the most widely used reagents to determine thiol groups and to determine the effects of thiol group modification on the properties of proteins (*1*). It reacts rapidly with most simple thiols and "exposed" thiol groups of proteins to produce an easily detected equivalent of 5-thio-2-nitrobenzoate (TNB) (Scheme 1). Slow reaction or failure to react is usually attributed to the inaccessibility of a thiol group or to its being in an anionic environment (*2*, *3*).

Mixed disulfides produced upon the reaction of DTNB with various thiols have also been used, in a few cases, to detect and to modify thiol groups of proteins

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[2] Abbreviations used: DTNB, 5,5′-dithiobis(2,2′-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate; ADNB, 5-(2-aminoethyl)dithio-2-nitrobenzoate; BSA, bovine serum albumin; ESA, equine serum albumin; CSA, canine serum albumin; MEA, 2-mercaptoethylamine; GSH, glutathione; CYS, cysteine; HOMO, homocysteine; TGA, thioglycolic acid; MPA, 3-mercaptopropionic acid; βME, β-mercaptoethanol; DTT, dithiothreitol.

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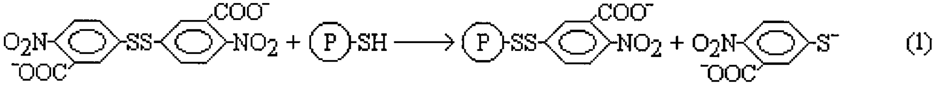


SCHEME 1

(Scheme 2). 5-(*n*-Octyl) dithio-2-nitrobenzoate, for example, reacts more rapidly than DTNB with some protein thiol groups and is thought to reflect their being in hydrophobic environments (*4–6*).

To determine whether other mixed disulfide derivatives of DTNB might be used to detect or characterize thiol groups in other unique environments, we synthesized and studied the reactions of 5-(2-aminoethyl)dithio-2-nitrobenzoate (ADNB) with a series of low-molecular-weight thiols and three closely related protein thiols. Because ADNB is a zwitterion, with little or no net charge under the conditions normally used, we anticipated that its reactions would be less sensitive to the ionic environments of the thiol groups with which it reacts. A comparison of its reactivity with a series of differently charged thiols to that of DTNB suggests that k_{ADNB}/k_{DTNB} ratios may be useful for characterizing the ionic environments of thiol groups in proteins.

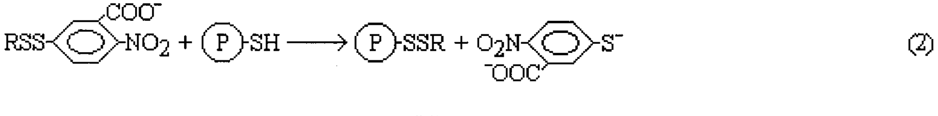
MATERIALS AND METHODS

Materials

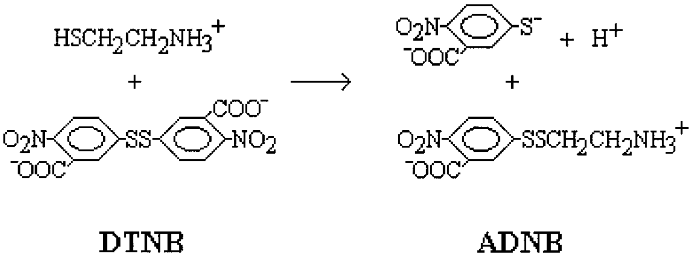
Thioglycolic acid (TGA), 3-mercaptopropoinic acid (MPA), and β-mercaptoethanol (βME) were obtained from Aldrich Chemical Co. 2-Mercaptoethylamine/HCl (MEA), glutathione (GSH), cysteine/HCl (Cys), homocysteine (HOMO), DTNB, bovine serum albumin (BSA, Fraction V), equine serum albumin (ESA, Fraction V), and canine serum albumin (CSA, Fraction V) were obtained from Sigma Chemical Co. To increase their thiol contents, serum albumins were treated at pH 7.4 for 30 min with a five-fold molar excess of dithiothreitol (DTT) and passed through a Sephadex G-50 column (*7*, *8*).

Preparation and Characterization of ADNB

DTNB was dissolved in H_2O to a final concentration of 0.126 M and adjusted to pH 7 with NaOH. One equivalent of MEA, also neutralized with NaOH, was added and mixed immediately (Scheme 3). After 1 h, the precipitate was collected by



SCHEME 2



(3)

SCHEME 3

vacuum filtration, washed three times with cold water, and dried. The resulting solid was redissolved in hot water and slowly cooled to room temperature. The light yellow crystals were collected, washed with cold water, and dried. The resulting product (60–70% yield) had a melting point of 215 to 221°C (decomp.), gave a single peak upon C-18 RP-HPLC and a single M + 1 peak (*m/z* 275) upon FAB mass spectroscopy. Its uv–visible spectrum (Fig. 1), determined with a HP 8452A diode array spectrophotometer, showed a peak at 328 nm with an extinction coeffi-

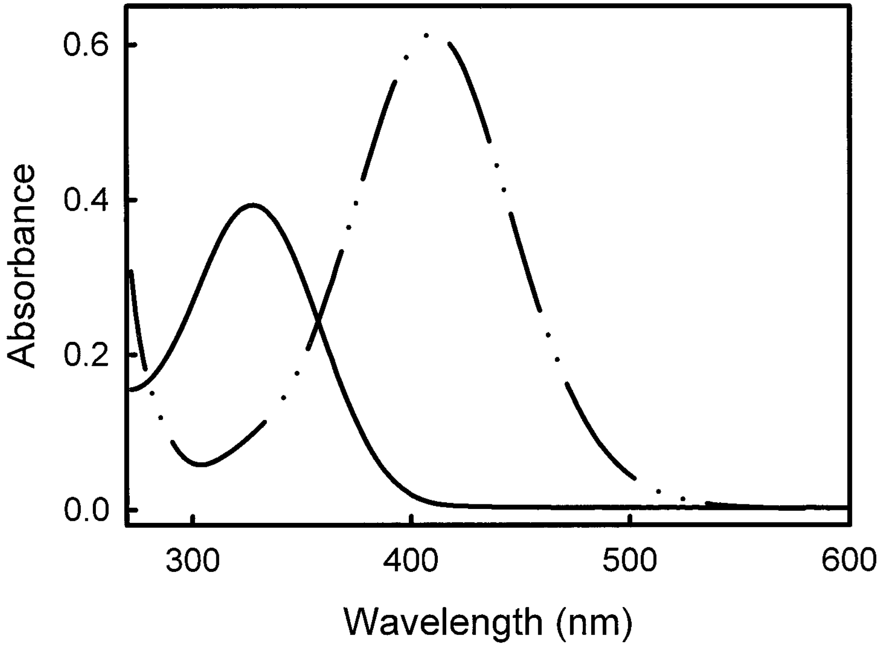


FIG. 1. The uv–vis spectrum of 45 μM ADNB in 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4 (———) and after reaction with 200 μM MEA (—··—).

TABLE 1
Second Order Rate Constants for the Reactions of ADNB and DTNB with Small Thiols[a]

RSH	k_{ADNB} (M^{-1} s^{-1})	k_{DTNB} (M^{-1} s^{-1})	$k_{\mathrm{ADNB}}/k_{\mathrm{DTNB}}$	Net charge
MEA	1020	4600	0.22	+1
HOMO	220	450	0.50	0
βME	110	220	0.50	0
CYS	550	940	0.58	0
GSH	300	270	1.11	−1
MPA	38	20	1.90	−1
TGA	94	31	3.03	−1

[a] Reactions were carried out using 5 μM of each thiol and a large excess of ADNB or DTNB in 10 mM MES, 1 mM EDTA, at pH 6.65. Reactions were followed by monitoring absorbance changes at 412 nm.

cient of ~8730 M^{-1} cm^{-1}. Upon addition of excess MEA, a new peak was formed with a maximum at 412 nm, corresponding to exactly one equivalent of TNB based on its absorbance at 412 nm (ε = 13,600 M^{-1} cm^{-1}) (*1*). ADNB stock solutions up to 5 mM were prepared by dissolving ADNB in a small amount of 1 M HCl and diluting it with 20 mM phosphate buffer at pH 6.65.

Kinetic Studies

Rate constants for the reactions of ADNB and DTNB with various thiols were determined as follows: 5 μM of thiol or thiol protein was added and rapidly mixed with excess (48 to 240 μM) of ADNB or DTNB in 10 mM MES buffer containing 1 mM EDTA at pH 6.65. The reactions were followed at 412 nm for at least eight half-lives. Pseudo-first-order rate constants, k_{obs}, were calculated according to the relationship $\ln(A_t/A_0) = -k_{\mathrm{obs}}t$. Values of k_{obs} at different concentrations of either ADNB or DTNB were plotted against reagent concentration to get second-order rate constants. Ionic strength dependencies were determined in 10 mM Mes, 1 mM EDTA, pH 6.65, with increasing amounts of NaCl. pH dependencies were determined using different buffers (sodium acetate at pH 5.3, sodium phosphate at pH 6 to 7, and Tris/HCl at pH 7 to 8) in the presence of 1 mM EDTA and an ionic strength of 0.02.

RESULTS AND DISCUSSION

ADNB, a zwitterionic mixed disulfide derivative of DTNB (Scheme 3), can be synthesized and purified in good yield due to its relatively low solubility at neutral pH. With little or no net charge at physiological pH, its reactivity with charged thiols and thiol groups located in the vicinity of charged groups of proteins are expected to differ from those of DTNB.

To evaluate the influence of charge on its reactivity, we compared its reactions with seven low-molecular-weight thiols with different charge characteristics to those of DTNB with the same thiols (Table 1). As with DTNB, reactions of ADNB