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A Kinetic Study of the Thiol–Disulfide Exchange Reaction between Aminothiols and 5,5′-Dithiobis(2-nitrobenzoic acid)

TOSHIHIKO OZAWA and AKIRA HANAKI*

National Institute of Radiological Sciences, Anagawa-4, Chiba 260, Japan

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The rate constants for the thiol–disulfide exchange, k_{SH}, and for the thiolate ion–disulfide exchange, k_{S^-}, between biologically relevant aminothiols, *i.e.*, cysteine, cysteamine and penicillamine, and 5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent, in aqueous solutions were determined by a spectrophotometric method. The apparent rate constant, k_1', increases with increase of pH under the experimental conditions used. The value of k_{S^-}, approximately 2×10^5 $\text{M}^{-1}\text{s}^{-1}$ except for penicillamine, appears to be independent of the pK_a (SH), and k_{SH} is three orders of magnitude smaller than k_{S^-}. The thiolate ion-disulfide exchange in penicillamine is one order of magnitude slower than in the other aminothiols. The results of the kinetic study indicate that one of the sulfur atoms of disulfide is attacked by the thiolate anion with subsequent cleavage of the S–S bond.

Keywords——Ellman's reagent; thiol–disulfide exchange; rate constant; cysteine; cysteamine; penicillamine; aminothiol

Because of the importance of thiol and disulfide groups in biochemistry,[1] substantial efforts have been made to design highly specific reagents for their determination.[2] One of the most widely used is 5,5′-dithiobis(2-nitrobenzoic acid), which is a water-soluble aromatic disulfide generally named Ellman's reagent.[3] The analytical procedure with Ellman's reagent, DTNB, is based on the exchange reaction to give the mixed disulfide and 2-nitrobenzoic acid, NTB, which is quantified by measurement of the optical absorption of the anion at 412 nm. The color development due to the reaction is very fast. The active species of the thiol for the exchange with DTNB has been considered to be the thiolate anion.[4–7] We have reexamined the thiol–disulfide exchange reaction in order to evaluate the kinetic parameters for the reactions of DTNB with the thiol and the thiolate anion. In this paper, the rate constants, k_{SH} and k_{S^-}, for the reaction are reported.

Experimental

Reagent-grade DTNB, cysteine, cysteamine, penicillamine and other aminothiols were used, and aqueous stock solutions in 0.1 M phosphate buffer were prepared freshly before the kinetic run. Water that had been deionized and doubly distilled in an all-glass apparatus was used throughout. Variation in pH was obtained by adding various amounts of conc. HCl or NaOH to the phosphate buffer solution. Ionic strength was adjusted to 0.11 with NaCl. Routinely, the concentrations of DTNB and aminothiols were 8.75×10^{-5} M and 6.25×10^{-5} M, respectively.

The reaction was initiated by mixing the solutions of DTNB and a thiol in a Union MX-7 rapid-mixing device and the optical density at 415 nm was followed thereafter. For the kinetic runs, a Hitachi RSP-2 rapid-scan spectrophotometer was used. Spectra at the initial and final states were measured on a Union SM-401 spectrophotometer. The reaction was performed at 25° under nitrogen.

The reaction product, NTB, has an extinction at 415 nm which is variable depending on pH in the range of 5—7. The molar extinction coefficients at various pHs are as follows; 9900 at pH 5.0, 11700 at pH 5.5, 12500 at pH 6.0, 13000 at pH 6.5 and 13600 above pH 7.0.

Results and Discussion

The thiol–disulfide exchange proceeds very fast and reaches equilibrium within a few seconds. The spectra at various times after mixing the solutions of DTNB and cysteine are shown in Fig. 1, where the spectra with $\lambda_{max}=325$ nm and with $\lambda_{max}=415$ nm are ascribable to DTNB and TNB, respectively. During the course of the reaction an isosbestic point is

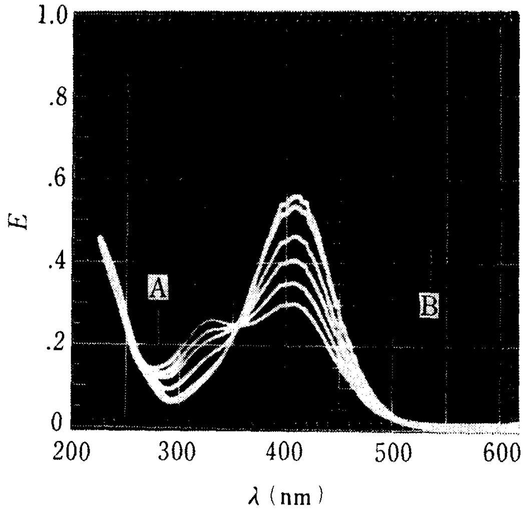

Fig. 1. Oscilloscope Traces of the Absorption Spectra during the Reaction of Cysteine with DTNB at pH 7.4 and 20°

[DTNB] = 7.0 × 10^{-5} M, [cysteine] = 4.3 × 10^{-5} M, Time; from the lowest at 415 nm, 0.15, 0.33, 1, 2, 4, 10 and 20 s.

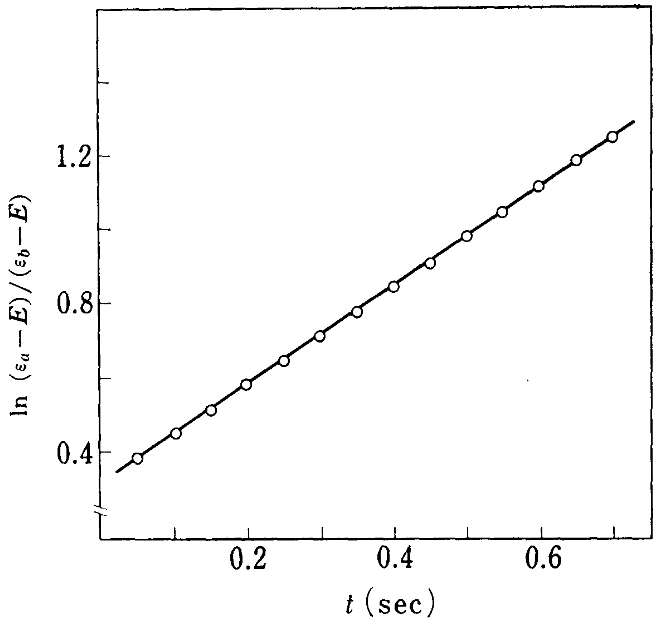

Fig. 2. Second-order Rate Plot for the Reaction of Cysteamine and DTNB

[DTNB] = 8.75 × 10^{-5} M, [cysteamine] = 6.25 × 10^{-5} M, pH 8.0, T = 25°.

observed at 355 nm, which indicates the absence of spectral intermediates. The reaction was followed by recording the optical density at 415 nm.

The stoichiometric ratio of the reaction between the thiol and DTNB was 2 : 1 . The overall reaction for the exchange can be expressed as a successive two-step reaction as shown by eqs. (1) and (2);

$$\text{RSSR} + \text{R'SH} \xrightarrow{k_1'} \text{RSSR'} + \text{RS}^- + \text{H}^+ \tag{1}$$

$$\text{RSSR'} + \text{R'SH} \xrightarrow{k_2'} \text{R'SSR'} + \text{RS}^- + \text{H}^+ \tag{2}$$

where RSSR, R'SH, RSSR' and RS^- represent DTNB, the thiol, the mixed-disulfide and the TNB anion, respectively.

The rate constant for the thiol-disulfide exchange, which we intended to determine, is k_1'. The rate, $d[\text{RS}^-]/dt$, observed in terms of the increase of E_{415}, is first order in the thiol, TNB and the mixed-disulfide as given by eq. (3).

$$\frac{d[\text{RS}^-]}{dt} = k_1'[\text{RSSR}][\text{R'SH}] + k_2'[\text{RSSR'}][\text{R'SH}] \tag{3}$$

when [RSSR] > [R'SH], the contribution of the reaction (2) to the overall reaction rate is small and the rate expression can be approximated by eq. (4).

$$\frac{d[\text{RS}^-]}{dt} = k_1'[\text{RSSR}][\text{R'SH}] \tag{4}$$

Then, the second-order rate constant k_1' expressed by eq. (5)

$$k_1' t = \frac{1}{(a-b)} \times \ln \frac{b(\varepsilon_a - E)}{a(\varepsilon_b - E)} \tag{5}$$

is obtained as the slope of the straight line on plotting $\ln(\varepsilon_a - E)/(\varepsilon_b - E)$ against t. In eq. (5), a and b represent the initial concentrations of DTNB and the thiol, respectively, and E is the optical density at t sec. The apparent rate constants calculated at pH 7 are shown in the second column of Table I.

Table I. Rate Constants for the Thiol–Disulfide Exchange Reaction

Compound	k_1' (pH 7) $10^3\,\text{M}^{-1}\,\text{s}^{-1}$	E'_a (pH 7)[a] kcal/mol	k_{S^-} $10^5\,\text{M}^{-1}\,\text{s}^{-1}$	pK_a (SH)
Cysteine	3.60	12.8	1.60	8.53
Penicillamine	2.10	11.7	0.220	7.90
Cysteamine	7.50	10.7	2.30	8.35
Cysteine ethyl ester	12.5	10.7	2.40	7.45
N-Acetylcysteine	0.520	14.1	2.15	9.52
Mercaptopropionic acid	0.170	13.9	2.35	10.05

a) Energy of activation at pH 7.

The mechanism of the exchange reaction has been proposed to be nucleophilic attack of the thiolate ion on one of the sulfur atoms of the disulfide with subsequent or simultaneous expulsion of the fragment containing the other sulfur atom of the disulfide as a thiolate ion.[4–7] The unionized form of the thiol is considered to be unreactive.[5,6] Accordingly, the reaction is expected to be enhanced with increase of pH. As shown in Fig. 3, a plot of log k_1' against pH gives a straight line with a slope of approximately unity in the pH range far below the pK_a(SH), which suggests that proton ionization of the thiol group assists the exchange reaction.

In order to confirm the proposed reaction mechanism, we intended to determine the rate constants of thiol–disulfide exchange for the thiolate, k_{s^-}, and for the thiol, k_{SH}. If the thiolate ion is the reactive species, the value of k_{s^-} should be much larger than k_{SH}. The experimentally observed rate is the sum of the contribution of the thiol and the thiolate ion, and is given by eq. (6);

$$k_1'[\text{RSSR}][\text{R'SH}]_0 = (k_{S^-}[\text{R'S}^-] + k_{SH}[\text{R'SH}])[\text{RSSR}] \qquad (6)$$

where $[\text{R'SH}]_0$ indicates the total concentration of the thiol. The concentrations of R'S^- and R'SH are functions of $[\text{H}^+]$ as shown by eqs. (7) and (8).

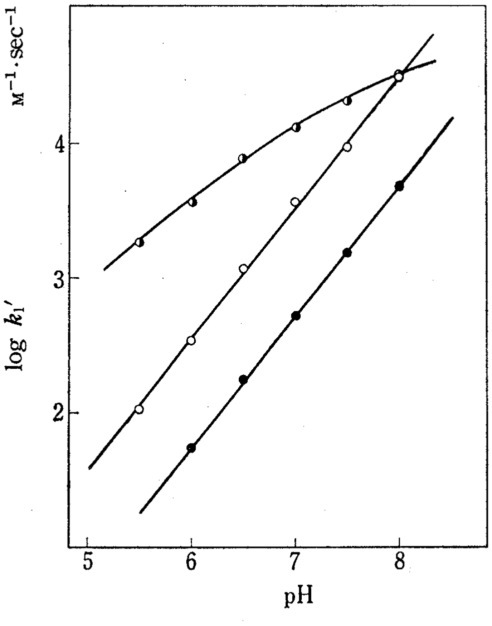

Fig. 3. pH Dependence of the Apparent Rate, k_1', at 25°

○—○; cysteine, ●—● ; N-acetylcysteine, ◑—◑ ; cysteine ethyl ester.

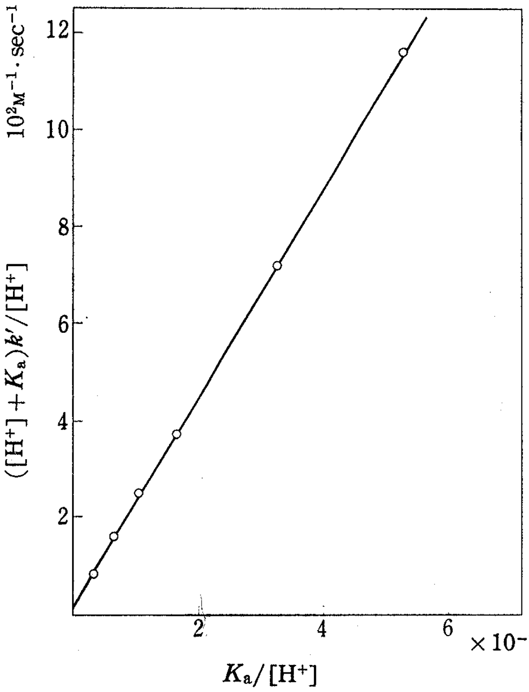

Fig. 4. Rate Plot for the Reaction of Penicillamine and DTNB

Experimental details are given in the text.

$$[R'S^-] = \frac{K_a[R'SH]_o}{([H^+]+K_a)} \qquad (7)$$

$$[R'SH] = \frac{[H^+][R'SH]_o}{([H^+]+K_a)} \qquad (8)$$

In eqs. (7) and (8), K_a represents the proton ionization constant of the SH group.[9,10] Then, by substituting eqs. (7) and (8) into eq. (6), the rate equation (9) is obtained.

$$\frac{([H^+]+K_a)}{[H^+]}k_i' = \frac{K_a}{[H^+]}k_{S^-} + k_{SH} \qquad (9)$$

The rate constant k_{s^-} can be obtained as the slope of the straight line upon plotting $([H^+]+K_a)k_i'/[H^+]$ against $K_a/[H^+]$, and k_{SH} can be obtained as the ordinate intercept. Fig. 4 shows an example of the plot obtained for the reaction of DTNB and penicillamine.

In aminothiols such as cysteine, which possesses a microscopic constant of proton ionization, the rate constant k_{s^-} is given eq. (10);

$$\frac{([H^+]+K_a+K_b)}{[H^+]}k_i' = \frac{K_a}{[H^+]}k_{S^-} + \frac{K_b}{[H^+]}k_{SH} + k_{SH^+} \qquad (10)$$

where k_{SH^+}, k_{s^-} and k_{SH} represent the rate constants for the reactions (11), (12) and (13), respectively.

$$H_3N^+\text{-}R'\text{-}SH + RSSR \xrightarrow{k_{SH^+}} NTB + \text{mixed-disulfide} \qquad (11)$$

$$H_3N^+\text{-}R'\text{-}S^- + RSSR \xrightarrow{k_{S^-}} NTB + \text{mixed-disulfide} \qquad (12)$$

$$H_2N\text{-}R'\text{-}SH + RSSR \xrightarrow{k_{SH}} NTB + \text{mixed-disulfide} \qquad (13)$$

The microscopic constants K_a and K_b correspond to the reactions (14) and (15), respectively.

$$H_3N^+\text{-}R'\text{-}SH \xrightarrow{K_a} H_3N^+\text{-}R'\text{-}S^- + H^+ \qquad (14)$$

$$H_3N^+\text{-}R'\text{-}SH \xrightarrow{K_b} H_2N\text{-}R'\text{-}SH + H^+ \qquad (15)$$

Since k_{SH} is very small as compared with k_{s^-} and can thus be neglected, the rate constant k_{s^-} is obtained as the slope of the straight line. A typical example is the reaction of cysteine and DTNB, as shown in Fig. 5.

The rate constants for the thiolate-disulfide exchange, k_s^-, are summarized in Table I. The values of k_s^- appear to be independent of the pK_a (SH) and they are in the range of 1.6—2.4×10^5 M^{-1} s^{-1} except for penicillamine. The thiolate ion of penicillamine reacts with DTNB at a slower rate, one order of magnitude smaller than the other rates. The two β-methyl groups of penicillamine probably interfere sterically with the nucleophilic attack of the thiolate anion on one sulfur atom of the disulfide. The rate constant for the thiol-disulfide exchange, k_{SH}, is three orders of magnitude smaller than k_{s^-}, which indicates that the sulfur atom of the disulfide is attacked preferentially by the thiolate anion with subsequent cleavage of the disulfide bond.

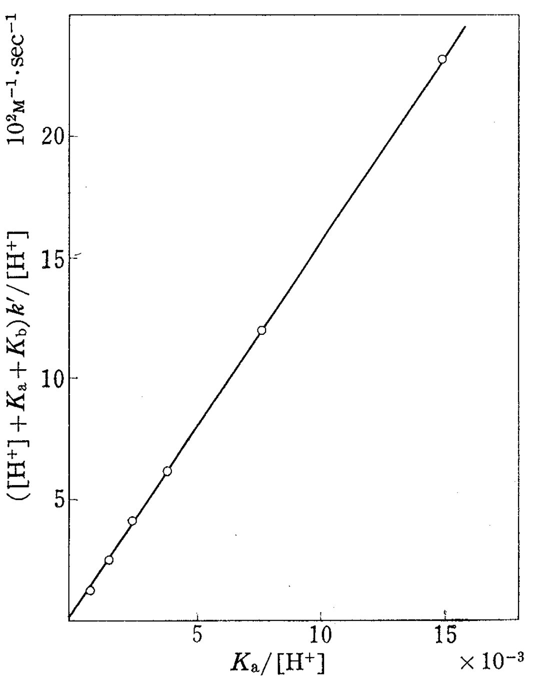

Fig. 5. Rate Plot for the Reaction of Cysteine and DTNB

References and Notes

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