Hope, M. J., & Cullis, P. R. (1981) *Biochim. Biophys. Acta 640*, 82-90.
Hsu, M.-C., Scheid, A., & Choppin, P. W. (1981) *J. Biol. Chem. 256*, 3357-3562.
Hsu, M.-C., Scheid, A., & Choppin, P. W. (1983) *Virology 126*, 361-369.
Impraim, C. C., Foster, K. A., Micklem, K. J., & Pasternak, C. A. (1980) *Biochem. J. 186*, 847-860.
Ishida, N., & Homma, M. (1978) *Adv. Virus Res. 23*, 349-383.
Janoff, A. S., Carpenter-Green, S., Weiner, A. L., Seibold, J., Weissmann, G., & Ostro, M. J. (1983) *Clin. Chem. (Winston-Salem, N.C.) 29*, 1587-1592.
Jensen, J. W., & Schutzbach, J. S. (1984) *Biochemistry 23*, 1115-1119.
Knutton, S., & Bachi, T. (1980) *J. Cell Sci. 42*, 153-167.
Kundrot, C. E., Spangler, E. A., Kendall, D. A., MacDonald, R. C., & MacDonald, R. I. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1608-1612.
Kundu, S. K. (1981) *Methods Enzymol. 72*, 185-204.
Lyles, D. S., & Landsberger, F. R. (1979) *Biochemistry 18*, 5088-5095.
Maeda, Y., Kim, J., Koseki, I., Mekada, E., Shiokawa, Y., & Okada, Y. (1977) *Exp. Cell Res. 108*, 95-106.
Markwell, M. A. K., Svennerholm, L., & Paulson, J. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5406-5410.
Neurath, A. R., Vernon, S. K., Hartzell, R. W., Wiener, F. P., & Rubin, B. A. (1973) *J. Gen. Virol. 19*, 21-36.
Nir, S., Bentz, J., Wilschut, J., & Duzgunes, N. (1983) *Prog. Surf. Sci. 13*, 1-124.
Oku, N., Inoue, K., Nojima, S., Sekiya, T., & Nozawa, Y. (1982) *Biochim. Biophys. Acta 691*, 91-96.
Papahadjopoulos, D., Jacobson, K., Nir, S., & Isac, T. (1973) *Biochim. Biophys. Acta 311*, 330-348.
Peretz, H., Toister, Z., Laster, Y., & Loyter, A. (1974) *J. Cell Biol. 63*, 1-11.
Richardson, C. D., & Choppin, P. W. (1983) *Virology 131*, 518-532.
Richardson, C. D. Scheid, A., & Choppin, P. W. (1980) *Virology 105*, 205-222.
Shimizu, K., & Ishida, N. (1975) *Virology 67*, 427-437.
Siegel, D. P. (1984) *Biophys. J. 45*, 399-420.
Skipski, V. P. (1975) *Methods Enzymol. 35*, 396-425.
Tomasi, M., D'Agnolo, G., & Ontecucco, C. (1982) *Biochim. Biophys. Acta 602*, 339-344.
Umeda, M., Nojima, S., & Inoue, K. (1983) *J. Biochem. (Tokyo) 94*, 1955-1966.
Verkleij, A. J., van Echteld, C. J. A., Gerristen, W. J., Cullis, P. R., & de Kruijff, B. (1980) *Biochim. Biophys. Acta 600*, 620-624.
Wu, P.-S., Ledeen, R. W., Udem, S., & Isaacson, Y. A. (1980) *J. Virol. 33*, 304-310.
Wyke, A. M., Impraim, C. C., Knutton, S., & Pasternak, C. A. (1980) *Biochem. J. 190*, 625-638.

Molecular Basis of Superreactivity of Cysteine Residues 31 and 32 of Seminal Ribonuclease

Augusto Parente,[‡] Bruce Merrifield,[§] Giuseppe Geraci,[∥] and Giuseppe D'Alessio*[,‡]

Dipartimento di Chimica Organica e Biologica and Istituto di Biologia Generale e Genetica, University of Naples, I-80134 Napoli, Italy, and Rockefeller University, New York, New York 10021-6399
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ABSTRACT: The molecular basis of the high reactivity toward reducing agents of intersubunit disulfides at positions 31 and 32 of dimeric bovine seminal ribonuclease was investigated by studying in the monomeric enzyme the fast reaction kinetics with disulfides of the adjacent cysteine-31 and -32, exposed by selective reduction of the intersubunit disulfides. Negatively charged and neutral disulfide reagents were used for measuring the thiol reaction rates at neutral pH. The kinetics studied as a function of pH permitted us to define pK values for the thiols of interest and indicated the possibility of determining pK values of SH groups in proteins indirectly by measuring the kinetics of reactivity of the SH groups with a disulfide reagent. The results were compared with those obtained under identical conditions with synthetic thiol peptides and model compounds. The data indicate that the superreactivity of intersubunit disulfides of seminal ribonuclease is matched by the high reactivity at neutral pH of adjacent cysteine residues 31 and 32, as compared to all small thiol compounds tested. The synthetic hexapeptide segment of seminal ribonuclease Ac-Met-Cys-Cys-Arg-Lys-Met-OH, which includes the two cysteine residues of interest, was even more reactive. These data, and the other results reported in this paper, led to the conclusion that the superreactivity at neutral pH of cysteine residues at positions 31 and 32 of bovine seminal ribonuclease is primarily dependent on the nearby presence of positively charged groups, particularly the ϵ-NH_2 of lysine-34, and is influenced by the adjacency of the two thiols and by the protein tertiary structure.

One of the main structural features of bovine seminal RNase is the presence of two interchain disulfide bridges linking the half-cystines at adjacent positions 31 and 32 of the two subunit chains (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975). There is an intriguingly high reactivity of these disulfides (D'Alessio et al., 1975) compared with that of the intrachain disulfides, four per subunit. Monomer $\rightleftharpoons$ dimer conversions may have physiological significance, as the cata-

[‡] Dipartimento di Chimica Organica e Biologica, University of Naples.
[§] Rockefeller University.
[∥] Instituto di Biologia Generale e Genetica, University of Naples.

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lytic activity of the dimeric enzyme was found to be modulable (Piccoli et al., 1982), while that of the dissociated monomer is not (Piccoli & D'Alessio, 1984). It should be noted that cleavage and re-formation of disulfides may be subjected to fine control by specific enzymes or by the cell redox state, which, in its turn, may be regulated.

The problem of the superreactivity of seminal RNase intersubunit disulfides was approached by studying in the monomeric enzyme the fast reaction kinetics of cysteines at positions 31 and 32, exposed by selective reduction of the parent disulfides (D'Alessio et al., 1975). The experiments were performed both with negatively charged Nbs_2[1] and with neutral DTP as reagents, in order to assess the influence of the reagent charge on the reaction. The results were compared with those obtained by investigating the reaction kinetics of (i) a synthetic peptide including two adjacent cysteines and corresponding to residues 29–34 of the subunit chain of seminal RNase, (ii) analogues of this peptide obtained through selective modifications of its charged residues, and (iii) appropriately selected simple thiol compounds, used as reference molecules. This experimental approach was selected so as to widen the scope of the research and contribute to the more general problem of cysteine reactivity in proteins (Liu, 1977; Torchinsky, 1981).

Materials and Methods

Enzymes. BS RNase was prepared from bull semen or from bull seminal vesicles as described (D'Alessio et al., 1972a; De Prisco et al., 1972). A faster procedure was also used (Tamburrini et al. submitted for publication) consisting of a single chromatographic run on (carboxymethyl)cellulose of seminal plasma. Monomeric BS RNase selectively reduced at the intersubunit disulfides was prepared by limited reduction with DTT as previously described (D'Alessio et al., 1975).

Peptides. L-Cysteinylcysteine, synthesized as previously described (Capasso et al., 1980), was generously supplied by Dr. S. Capasso (Istituto Chimico, University of Naples). Hexapeptide P(29–34), corresponding to the sequence of region 29–34 of seminal RNase subunit, was prepared according to published procedures (Erickson & Merrifield, 1976). In brief, methionine residues were protected as methionine sulfoxide; the amino acids were used with their α-NH_2 groups blocked with a *tert*-butyloxycarbonyl group; the other protecting groups were 4-methoxybenzyl for the cysteine residues, 4-toluenesulfonyl for the arginine, and 2-chlorobenzyloxycarbonyl for the lysine residues. Deprotection of the main chain was carried out with trifluoroacetic acid, followed by neutralization with diisopropylethylamine and coupling by activation with dicyclohexylcarbodiimide. The α-amino group of the assembled peptide still attached to the copoly(styrene–1% divinylbenzene) resin was deprotected and acetylated with acetic anhydride in pyridine. Deprotection and cleavage of the peptide from the resin was achieved by treatment with HF containing 10% anisole, followed by extraction in 5% acetic acid. Minor contaminants, detected by paper electrophoresis, were removed by gel filtration of the peptide, previously reduced with DTT, on a column of Sephadex G-25 equilibrated and eluted with 1% acetic acid. Reduction of methionine sulfoxides was accomplished by 1% mercaptoacetic acid at pH 8. After 6 days, 96% of the sulfoxide was found to be reduced by amino acid analysis. After a final gel filtration on Sephadex G-25, the amino acid composition of the peptide, oxidized with performic acid, was as reported in Table I.

Table I: Amino Acid Compositions of Synthetic Hexapeptide Fragment of BS RNase P(29–34) and of Its Derivatives

amino acid	peptide P(29–34)	[N^{ϵ}-Ac-Lys^5]-P(29–34)	[N^{δ}-Ac-Orn^4,-N^{ϵ}-Ac-Lys^5]-P(29–34)
Met	2.11[a]	1.99	1.97
Cys	1.87[b]	ND[c]	ND
Arg	0.93	1.07	0
Orn			1.02
Lys	1.00	1.00	1.00

[a] Determined as methionine sulfone after performic acid oxidation. [b] Determined as cysteic acid after performic acid oxidation. [c] ND, not determined.

[N^{ϵ}-Ac-Lys^5]P(29–34), a derivative of peptide P(29–34), was obtained by acetylating the ϵ-amino group of Lys-5 according to Morris et al. (1971). The peptide, dried from 1% triethylamine, was reacted with acetic anhydride in methanol for 2 min at room temperature. Excess reagent was removed under vacuum.

[N^{δ}-Ac-Orn^4,N^{ϵ}-Ac-Lys^5]P(29–34), another derivative of P(29–34), was prepared by converting the arginine residue of the parent peptide into ornithine, followed by acetylation of the amino groups of Orn-4 and Lys-5. Conversion of arginine to ornithine was obtained as described by Morris et al. (1973), by heating the peptide in hydrazine at 75 °C; the reaction mixture was then cooled and excess reagent removed under vacuum. The reaction product, also containing unreacted material, was acetylated as described above. The peptide of interest was then purified by high-voltage paper electrophoresis at pH 6.5 in pyridine–acetate buffer. Peptides were located by the chlorination method (Reindel & Hoppe, 1954) and eluted with 0.2 M ammonia solution. Amino acid analyses of the peptide derivatives, carried out on a LKB 4400 autoanalyzer, gave the compositions reported in Table I.

Kinetic Analysis. Titrations of the SH groups of the various samples were performed with Nbs_2 in a Zeiss PMQ II spectrophotometer. Reaction kinetics were followed in a Gibson Durrum stopped-flow spectrophotometer equipped with a 2-cm light-path cuvette at 20 ± 0.1 °C. Sample concentration was 20 μM in SH groups while Nbs_2 and DTP were 1 mM before mixing in the stopped-flow apparatus. The samples, in 20 mM KCl containing 2 mM EDTA, were diluted to the final concentration with 50 mM Tris, 50 mM CAPS, and 50 mM citrate and adjusted to the desired pH values with KOH and to the same conductivity values of 9 $m\Omega^{-1}$, corresponding to a ionic strength value of 0.15, with KCl. Nbs_2 was dissolved in 1 mM Tris-HCl, pH 8, and DTP in 95% ethanol; they were diluted before each kinetic run to 1 mM in the experiment buffer. Reactions were followed at 412 nm when the reagent was Nbs_2 and 343 nm when DTP was used. The extinction coefficients used were ϵ_{412} = 13 600 cm^{-1} M^{-1} for the thionitrobenzoate anion, ϵ_{343} = 7060 cm^{-1} M^{-1} for thiopyridine, and ϵ_{281} = 9370 cm^{-1} M^{-1} for DTP.

Results and Discussion

The time course of the reaction of the sulfhydryls of cysteine residues 31 and 32 of monomeric seminal RNase with Nbs_2 is illustrated in Figure 1, where it is compared with that of the sulfydryl group of free cysteine. The reactions, carried out at pH 7, follow a pseudo-first-order rate equation. The two SH groups of monomeric BS RNase are not distinguishable under the conditions of the experiment and react much faster than the SH group of cysteine.

In addition to Nbs_2, a negatively charged disulfide reagent, also DTP, a reagent with no charge under the conditions of

[1] Abbreviations: BS RNase, bovine seminal ribonuclease; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); DTP, 2,2′-dithiopyridine; DTT, 1,4-dithiothreitol; 2-ME, 2-mercaptoethanol; Ac, acetyl; Na_2EDTA, sodium ethylenediaminetetraacetate; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; P(29–34), Ac-Met-Cys-Cys-Arg-Lys-Met-OH; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

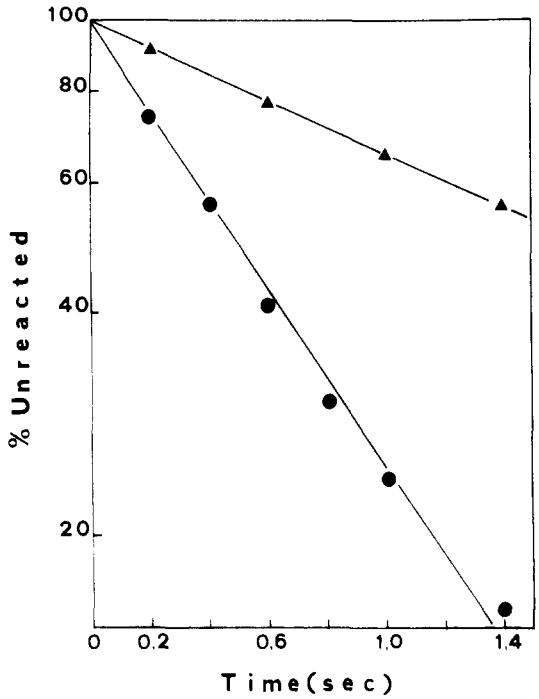


FIGURE 1: Time course of reactions of cysteine (▲) and of seminal RNase cysteine residues 31 and 32 (●) with Nbs_2 at pH 7.0. The ordinate gives the percent of SH groups remaining unreacted.

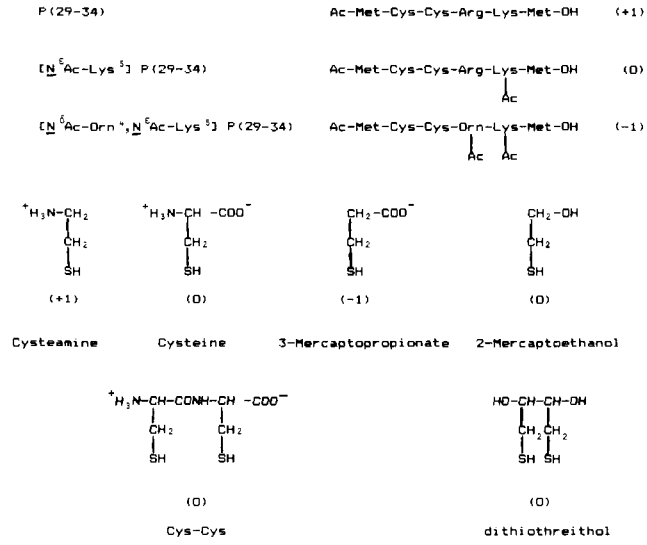
Table II: Second-Order Rate Constants of Protein Thiols and of Model Thiols with Nbs_2 and DTP at pH 7.0, 20 °C

thiol compd	k_{obsd} ($\times 10^{-3}$ M^{-1} s^{-1}) of disulfide reagents	
	Nbs_2	DTP
BS RNase (monomeric)	3.46	1.92
peptide P(29–34)	9.02	4.50
[N^{ϵ}-Ac-Lys^5]P(29–34)	3.93	3.03
[N^{δ}-Ac-Orn^4,N^{ϵ}-Ac-Lys^5]P(29–34)	1.54	1.61
cysteine	0.80	0.70
cysteinylcysteine	0.77	0.63
2-mercaptoethanol	0.16	0.07
dithiothreitol	0.60	0.26
cysteamine	1.98	1.12
mercaptopropionic acid	0.04	<0.04

the experiment, was used. The data, reported in Table II, are compared with the data obtained under identical conditions with model peptides and small thiol compounds. Chart I shows the structures of these compounds, hence the rationale for their choice, as well as the amino acid sequences of the model peptide P(29–34), corresponding to the sequence of BS RNase subunit that includes the two cysteine residues of interest and of its derivatives, where one or both positive charges have been eliminated.

With uncharged DTP, cysteine residues 31 and 32 of monomeric BS RNase reacted at a slower rate than with negatively charged Nbs_2. However, with both reagents, the rates of the two thiols were higher than any of those of the small model thiols tested. The synthetic peptide P(29–34), with a net charge of 1+, was found to react with both disulfide reagents at rates even higher than those of monomeric RNase. This may not be surprising, as the peptide inserted in the protein molecular organization should have a much reduced mobility with respect to the free peptide and, in contrast with the protein, the peptide thiols can be approached by the reagents from all directions. Furthermore, in the protein the sequence segment 29–34 is part of an α-helix cylinder where the two cysteine side chains cannot interact with one another

Chart I[a]



[a] The net charge is shown in parentheses.

(Capasso et al., 1983), while the free peptide may well assume different structures. In fact, when peptide P(29–34) was reduced with excess DTT and then fully reoxidized in air, it was found (data not shown) that about 50% of the reaction product was the dimeric peptide, maintained by the two interpeptide disulfides. The remaining was monomeric, with the two adjacent half-cystine residues forming an intrapeptide disulfide. In the latter case, an α-helix conformation has clearly to be ruled out.

The N^{ϵ}-Ac-Lys^5 derivative of peptide P(29–34), with no net charge, reacted with both disulfide reagents at a slower rate than the unmodified peptide (see Table II). The decrease in rate was however much more marked with Nbs_2 than with DTP. The N^{δ}-Ac-Orn^4,N^{ϵ}-Ac-Lys^5 derivative (with net charge 1−) reacted even more slowly with the disulfide reagents. Interestingly, the thiol groups of this peptide reacted at almost the same rate with both Nbs_2 and DTP. All these data clearly indicate that the presence of positively charged groups in the vicinity of a thiol group has a strong effect in enhancing thiol reaction rates. However, it should be noted that the reaction rate of the two cysteine residues of interest was found to be still higher than that of free cysteine even in the derivative where all positively charged groups had been eliminated. This indicates that the reactivity of the two adjacent cysteine residues is influenced also by other factors, including steric hindrance due to the modifying acetyl group.

The possibility that adjacency is a determining factor was investigated by comparing the reaction rates with Nbs_2 and DTP of two monothiols, Cys and 2-ME, with those of the corresponding dithiols: Cys-Cys and DTT (see Chart I). An enhancing effect of adjacency on the reaction rate is evident when the data obtained with 2-ME are compared to those of DTT (see Table II). In this case, the monothiol reacts at a rate about one-fourth that of the dithiol with both disulfide reagents. This shows that the adjacency of thiol groups is a factor promoting higher reaction rates.

Higher reducing rates of dithiols, with respect to the corresponding monothiols, have been previously correlated with the ring size of the cyclic disulfides produced upon oxidation of the dithiols (Szajewski & Whitesides, 1980). These studies revealed also that the effect is attributable to the higher forward rate of reaction step 2, compared to that of reaction step 2a:

$$RSSR + HSR_1SH \rightleftharpoons RSSR_1SH + RSH \quad (1)$$

$$RSSR_1SH \rightleftharpoons \overbrace{SR_1S} + RSH \quad (2)$$

$$RSSR + HSR_1 \rightleftharpoons RSSR_1 + RSH \quad (1a)$$

$$RSSR_1 + HSR_1 \rightleftharpoons R_1SSR_1 + RSH \quad (2a)$$

Under our experimental conditions, reaction kinetics depended only on the forward rates of steps 1 and 1a. Thus, the influence of reaction steps 2 and 2a can be excluded in our experiments.

The lack of a difference in reactivity between the two compounds Cys and Cys-Cys seems to oppose the possibility that adjacency is a promoting factor in this case (see Table II). However, the absence of charged groups on 2-ME and DTT may suggest an explanation (i) for the apparent lack of effect of thiol adjacency in the pair Cys/Cys-Cys and (ii) for the high reaction rate of the two adjacent cysteines of interest even in the neutral hexapeptide where all positive charges are neutralized. The positive and the negative charges (α-NH_3^+ and α-COO^-), which in Cys and in Cys-Cys are in the vicinity of the thiol groups, may influence their reactivity so as to mask the enhancing effect of adjacency in Cys-Cys. In the case of the N^{δ}-Ac-Orn[4],N^{ϵ}-Ac-Lys[5] derivative, no charged groups are present near the adjacent thiols; thus in this case, as in the case of DTT, the enhancing effect on the reaction rate of thiol adjacency would become evident. The importance of single charges on the reactivity of the thiols was in fact clearly shown by the data obtained with cysteamine and mercaptopropionate, which can be considered as positively and negatively charged derivatives, respectively, of 2-ME (see Chart I). The reaction rate of mercaptopropionate with both disulfide reagents was one-hundredth that of cysteine.

Charge effects on thiol reactivity were further characterized by studying the thiol reaction rates as a function of pH. The plot of the apparent second-order rate constants for the SH group vs. pH has a characteristic pattern (Lindley, 1960) resembling that of the titration curve of an acidic group, with the midpoint pH value corresponding to its pK values. That the pK value of an SH group can be obtained from kinetic measurements is easily derived assuming that the SH group exists in the following equilibrium:

$$R\text{-}SH \underset{k''}{\overset{k'}{\rightleftharpoons}} R\text{-}S^- + H^+ \qquad K = k'/k'' \quad (3)$$

If R–S$^-$ reacts with rate constant k_1 and R–SH with rate k_2, such that k_1, $k_2 \ll k'$, k'', then

$$v_{tot} = -d[R\text{-}SH]/dt - d[R\text{-}S^-]/dt = k_1[R\text{-}S^-] + k_2[R\text{-}SH] \quad (4)$$

and when $k_2 = 0$, as in the case that the reaction species is only R–S$^-$, then

$$-d([R\text{-}SH] + [R\text{-}S^-])/dt = k_{obsd}([R\text{-}SH] + [R\text{-}S^-]) \quad (5)$$

$$[R\text{-}S^-]/[R\text{-}SH] = K/[H^+] \quad (6)$$

Since combining eq 4 and 5 and substituting with eq 6

$$k_{obsd} = (k_2 + k_1K/[H^+])/(1 + K/[H^+]) \quad (7)$$

Given $k_2 = 0$

$$k_{obsd} = (k_1K/[H^+])/(1 + K/[H^+]) \quad (8)$$

The measured rate constant k_{obsd} has values between zero and k_1 depending on the pH, and in particular, when $K = [H^+]$, $k_{obsd} = k_1/2$ and the midpoint of the function k_{obsd} vs. $[H^+]$ is indeed the pK of the group.

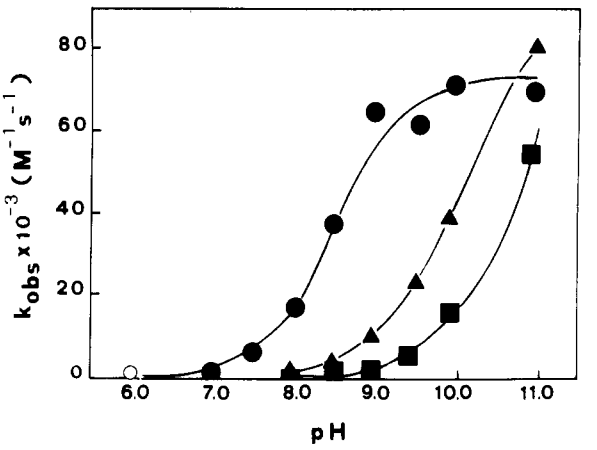


FIGURE 2: Plots of the second-order rate constants vs. pH for the reaction of Nbs_2 with cysteamine (●), 2-mercaptoethanol (▲), and 3-mercaptopropionic acid (■).

Srajewski & Whitesides (1980), while studying thiol–disulfide interchange reactions in proteins, have derived the expression

$$k_1 = (10^{pK-pH} + 1)k_{obsd} \quad (9)$$

which relates as eq 8 does the observed rate constant to the microscopic rate constant and to the pK values of the thiolate. Although based on different assumptions, eq 9 can be easily converted into eq 8. The possibility of determining indirectly pK values of SH groups through kinetic measurements was first indicated by Shaked et al. (1980), who measured rates of reduction with DTT of disulfide derivatives of the thiols of interest. The procedure described here may be applied directly to the thiols of interest, as it does not require their derivatization.

Plots of the second-order rate constants vs. pH are shown in Figure 2 for three model compounds: 2-ME and its two "derivatives" cysteamine and mercaptopropionate (see Chart I). The pK values as determined with the present procedure compare well with values (shown in parentheses) previously obtained with titrimetric methods (Danehy & Noel, 1960): mercaptoacetic acid, 10.4 (10.20); 2-ME, 9.75 (9.48); cysteamine, 8.45 (8.35). The pK values of the SH groups are thus found at a higher pH value when a negatively charged group is adjacent to the SH group and at a lower pH when the adjacent group is positively charged.

In Figure 3 we present the data on the synthetic P(29–34), peptide and on the two derivatives with net charges equal to 0 and to 1–. While the neutralization of the Lys charge (see Table I) shifts the apparent pK of the SH groups to a higher value, neutralization of both the Arg and the Lys positive charges has no additional influence on the apparent pK value but does further lower the rate constant. This indicates that in a peptide fragment and, very likely, in the protein as well the two charged residues have a different influence on the SH reactivity of Cys-31 and -32.

The effects of pH appear more complex in the monomeric enzyme, as shown in Figure 4. In this case, a discontinuity of the dependence of the reaction rate constant upon pH occurs between pH 8.5 and pH 9.5. At values lower than pH 8.5, the function is similar to that typical of cysteamine, while at pH values higher than 9.5 the pattern is similar to that typical of 2-ME. The values of the apparent rate constants in these intervals of pH derive from monophasic time courses that are faster at increasing pH values. At values between pH 8.5 and 9.5, two phases are clearly evident in the reaction progress curves, corresponding to two rate constants differing by a

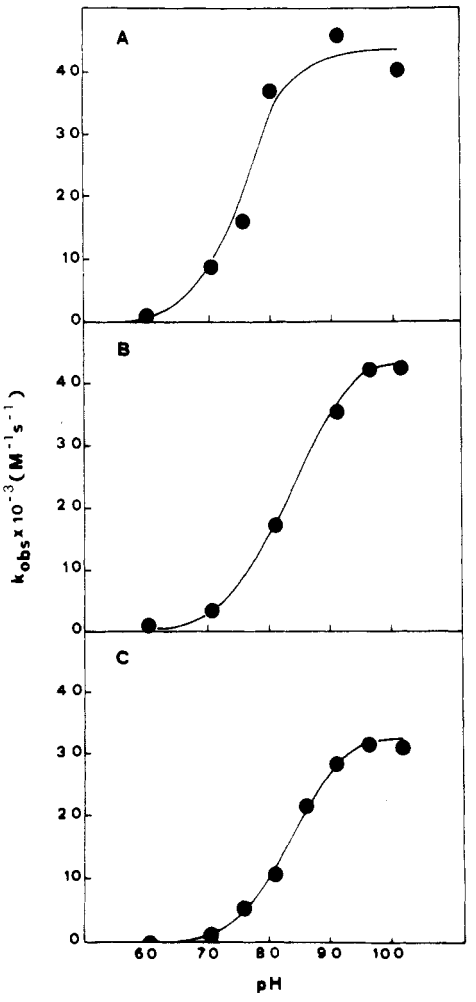


FIGURE 3: Plots of the second-order rate constants vs. pH for the reaction of Nbs_2 with (A) peptide P(29–34), (B) N^{ϵ}-Ac-Lys^5 derivative of peptide P(29–34), and (C) N^{δ}-Ac-Orn^4, N^{ϵ}-Ac-Lys^5 derivative of peptide P(29–34).

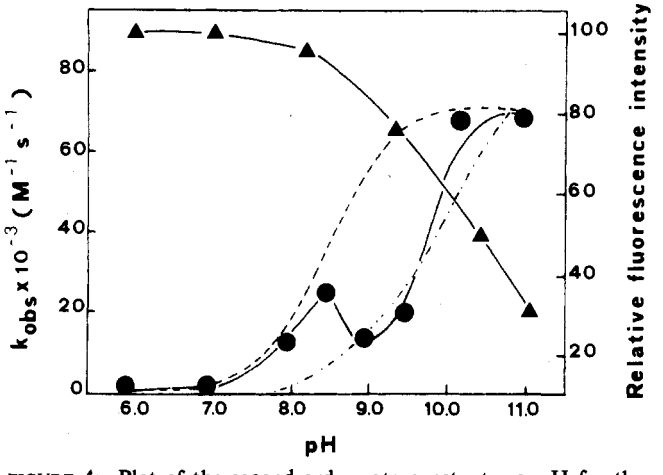


FIGURE 4: Plot of the second-order rate constants vs. pH for the reaction of Nbs_2 with cysteine residues 31 and 32 of monomeric BS RNase (●). The profiles of the pH dependence of the second-order rate constants for the reaction of Nbs_2 with cysteamine (– –) and with 2-ME (–·–) are derived from the data of Figure 2. The pH dependence of the relative fluorescence intensity of BS RNase (Grandi et al., (1979) is also shown (▲).

factor of about 10. The percent reaction corresponding to the fast and to the slow phases depends on pH, and as shown in Figure 5, their relative amounts are almost equal at pH 9.0. In Figure 4 are reported only the values of the rate constant corresponding to the fast phases.

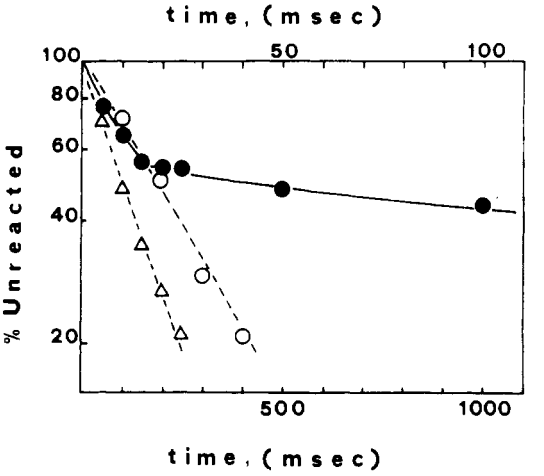


FIGURE 5: Time courses of the reactions of cysteine residues 31 and 32 of monomeric BS RNase with Nbs_2 at different pH values. Upper abscissa scale is pH 10 (△); lower scale is pH 8 (○) and pH 9 (●). On the ordinate scale is the percent of SH groups remaining unreacted.

It has been reported that fluorescence spectroscopic analysis of the enzyme shows a constant emission intensity at 303 nm from pH 5 to pH 8, while above this pH value the quantum yield decreases sigmoidally reaching a new lower value (Grandi et al., 1979). This was interpreted to be a consequence of the relaxation of the protein structure in a new state, induced by the increase of pH (Grandi et al., 1979). The discontinuity of the second-order rate constant vs. pH for the SH reaction rate (Figure 4) is in line with this interpretation and suggests that the influence on the SH reactivity of a positively charged group(s) is lost in the monomeric enzyme when the protein structure is altered.

Cys-25 of papain was reported to have widely different pK values, depending on the experimental conditions. However, the values were obtained indirectly by measuring the rates of reduction by DTT of several mixed disulfide derivatives of the cysteine of interest (Shaked et al., 1980). Also, in that case, the transition was attributed to charge effects from a neighboring base.

CONCLUSIONS

The data we present are in good agreement with those reported in the literature on the factors affecting the reaction rate of SH groups in proteins and in model thiol compounds (Whitesides et al., 1977; Wilson et al., 1977, 1980; Srajewski & Whitesides, 1980; Shaked et al., 1980; Synder et al., 1981). They provide convincing information on the molecular bases of the superreactivity at neutral pH of cysteine residues 31 and 32 of monomeric BS RNase. Our data, and those of previous authors, indicate that, in principle, several factors have to be considered as potentially influencing the reactivity of thiols: (i) the electrostatic environment, particularly the presence of positively charged groups in the environment; (ii) in the case of protein thiols, the protein tertiary structure; (iii) inductive effects, exerted through the carbon backbone, on the thiolate sulfur; (iv) the adjacency of the thiol groups.

The latter factor has not been demonstrated to our knowledge in previous studies. On the basis of the results we report, it appears that adjacency of the thiols has a role in determining the high reactivity of the thiols at least in the case of DTT in the reaction with either Nbs_2 or the uncharged DTP, as compared to 2-ME. A similar effect appears to be operative for the adjacent cysteine residues in the derivative of peptide P(29–34) where all positive charges have been eliminated.

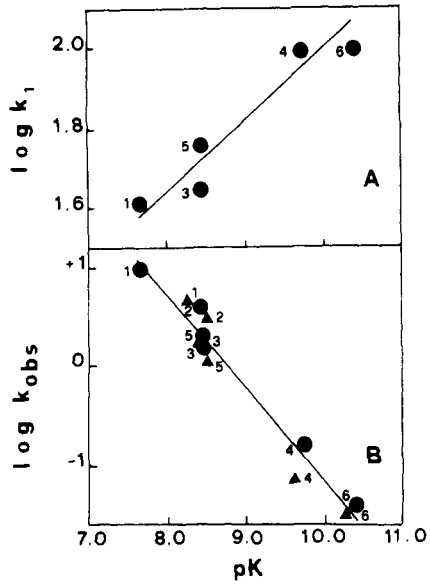


FIGURE 6: Rate constants for the reaction of thiol compounds with Nbs_2 (●) and DTP (▲) are plotted (A) as log k_1 and (B) as log k_{obsd} vs. the pK values calculated from the curves of Figures 2 and 3, using eq 8. The numbers refer to (1) synthetic peptide P(29–34), (2) N^{ϵ}-Ac-Lys^5 and (3) N^{δ}-Ac-Orn^4,N^{ϵ}-Ac-Lys^5 derivatives of peptide P(29–34), (4) 2-ME, (5) cysteamine, and (6) mercaptopropionic acid. The data point for [N^{ϵ}-Ac-Lys^5]P(29–34) has been omitted in (A). The lines were fitted through the points by a least-squares program.

The adjacency of the cysteine residues may have a direct role on their reactivity when they are in a small peptide but not when they are included in the structure of BS RNase. In this case, the side chains of the two cysteine residues project toward the outside of an α-helix cylinder (Capasso et al., 1983) and cannot interact one with the other.

Inductive effects have been shown to play a role on the reaction rate of thiol–disulfide interchange reactions (Freter et al., 1979). Although no evidence has been obtained in this study on their role in modulating the reactivity of the protein or peptide thiols, inductive effects cannot be excluded as a factor influencing the reactivity of Cys-31 and Cys-32 of BS RNase.

Previous authors (Whitesides et al., 1977; Wilson et al., 1977; Srajewskj & Whitesides, 1980) have found that rates of thiol–disulfide interchange, measured with Nbs_2 as a disulfide reagent, follow a Brønsted correlation with a β_{nuc} of approximately 0.5. The pK values of the thiols used in these studies were obtained from titration curves. Figure 6A shows a Brøsted plot, of log k_1 vs. thiol pK. The k_1 values were derived, using eq 8, from the k_{obsd} data listed in Table I and the approximate pK values computed from the plots of Figures 3 and 4. A satisfactory correlation is indeed observed (correlation coefficient 0.97), except for the peptide derivative [N^{ϵ}-Ac-Lys^5]P(29–34), which shows a strong deviation from the line fitted to the other data points with a least-squares program. The β_{nuc} value of 0.17 is lower than those reported for other protein thiols (Shaked et al., 1980). This may be due to the relatively narrow range of pK values and to the limited number of data points. No correlation was found with the data obtained for the same thiols but with DTT as a disulfide reagent (see Table I). The log k_1 data points scattered whether they were plotted vs. the pK values computed in this study or those of the literature. We have no explanation for this finding. However, both series of data, from the experiments with Nbs_2 and with DTT, gave good correlations between the log k_{obsd} and the thiols' pK values (see Figure 6B). As expected (Srajewskj & Whitesides, 1980), the closer the pK of the thiol was to the solution pH, the higher was the reactivity.

It has been reported that the electrostatic influence of charged residues in the environment of a cysteine SH group can affect by orders of magnitude the value of the reaction rate with a negatively charged disulfide at pH 7 (Synder et al., 1981). On the basis of our results, it appears that this effect, as evidenced by the data of Table II, is caused by a shift in the apparent pK of the SH group. This shift is clearly evident from the comparative analysis of the reactions of cysteamine, 2-mercaptoethanol, and mercaptopropionic acid, as well as of model peptides (see Figures 2 and 3). The pK shift is presumably caused by the formation of an internal ion pair, as suggested in a study of the reaction rate of bovine serum albumin with Nbs_2 (Wilson et al., 1980).

Arg-33 and Lys-34 are the basic residues that follow the two cysteines of interest in the amino acid sequence of BS RNase subunit (Suzuki et al., 1976). X-ray studies (2.5-Å resolution) on BS RNase crystals (Capasso et al., 1983) have revealed that the guanidinium group of Arg-33 is engaged in a salt linkage with Asp-14 and that the Lys-34 amino group does not point directly toward the two half-cystines. On the basis of these data, it may be suggested that an indirect effect of the two positively charged residues on the local pH is sufficient to stabilize the reactive thiolates. Another interpretation may be advanced by taking into account that the X-ray data were collected on dimeric BS RNase and that the protein undergoes a conformational change upon dissociation to monomers (Grandi et al., 1979; Parente et al., 1976). The possibility is that in the conformation of free monomer one or both of the two basic side chains (Arg-33 and Lys-34) exert a more direct effect on the thiol groups of Cys-31 and -32. However, a clear parallelism is evident between the high reactivity toward thiol reagents of the intersubunit disulfides of BS RNase (D'Alessio et al., 1975) and the high reactivity toward disulfide reagents of the cysteine residues involved in these intersubunit bridges.

Finally, the importance of the overall protein organization is especially stressed by the discontinuity in the pH curve of the rate constants of Cys-31 and -32 in the protein, which was not found when the reactivity of these cysteines was studied in the model peptides. Previous data of the literature may now be easily explained. The reaction rate of the β-93 SH group of human hemoglobin A with p-(hydroxymercuri)benzoate was found to be invariant in the pH interval between 6 and 9, as studied by stopped-flow measurements in the $\alpha_2\beta_2$ tetramer (Geraci & Parkhurst, 1973), while in the tetramer of β chains the expected increase was found (Geraci & Parkhurst, 1971). More recently, this type of influence on the reaction rate of the β-93 SH group has been detected by using selected variants of human hemoglobin (Hallway et al., 1980). Also, the superreactivity of Cys-110 of histone H3 from chicken erythrocytes has been reported to depend on the tertiary structure of the protein, where an artifactual cluster of positive charges around the SH group is formed, rather than on a stable local microenvironment (Bode et al., 1982). It seems reasonable to conclude that in proteins local electrostatic effects and inductive effects can be enhanced or diminished by the tertiary and quaternary structures of the molecule so that each protein thiol group is a case in itself, presumably formed under the influence of the stabilization of an effective functional structure for survival of the organism.

Registry No. RNase, 9001-99-4; Nbs_2, 69-78-3; DTP, 2127-03-9; DTT, 3483-12-3; 2-ME, 60-24-2; P(29–34), 94732-83-9; [N^{ϵ}-Ac-Lys^5]P(29–34), 94732-84-0; [N^{δ}-Ac-Orn^4,N^{ϵ}-Ac-Lys^5]P(29–34),

94732-85-1; Cys, 52-90-4; Cys-Cys, 18048-87-8; cysteamine, 60-23-1; mercaptopropionic acid, 107-96-0.

References

Bode, J., Wingender, E., & Plank, K. H. (1982) *Eur. J. Biochem. 123*, 23-28.

Capasso, S., Mattia, C. A., Mazzarella, L., & Puliti, R. (1980) *J. Chem. Soc., Perkin Trans. 2*, 1297-1300.

Capasso, S., Giordano, F., Mattia, C. A., Mazarella, L., & Zagari, A. (1983) *Biopolymers 22*, 327-332.

D'Alessio, G., Floridi, A., De Prisco, R., Pignero, A., & Leone, E. (1972) *Eur. J. Biochem. 26*, 153-161.

D'Alessio, G., Malorni, M. C., & Parente, A. (1975) *Biochemistry 14*, 1116-1121.

Danehy, J. P., & Noel, C. J. (1960) *J. Am. Chem. Soc. 82*, 251-2515.

De Prisco, R., Farina, B., & Leone, E. (1972) *Boll—Soc. Ital. Biol. Sper. 48*, 1111-1114.

Di Donato, A., & D'Alessio, G. (1973) *Biochem. Biophys. Res. Commun. 55*, 919-928.

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins 2*, 255-527.

Freter, R., Pohl, E. R., Wilson, J. M., & Hupe, D. J. (1979) *J. Org. Chem. 44*, 1771-1774.

Geraci, G., & Parkhurst, L. J. (1971) *Atti Accad. Naz. Lincei, Cl. Sci. Fis., Mat. Nat., Rend. 50*, 12-16.

Geraci, G., & Parkhurst, L. J. (1973) *Biochemistry 12*, 3414-3418.

Grandi, C., D'Alessio, G., & Fontana, A. (1979) *Biochemistry 18*, 3413-3420.

Hallaway, B. E., Hedlund, B. E., & Benson, E. S. (1980) *Arch. Biochem. Biophys. 203*, 332-342.

Lindley, H. (1960) *Biochem. J. 74*, 577-584.

Liu, T. Y. (1977) *Proteins 3*, 239-402.

Morris, H. R., Williams, D. H., & Ambler, R. P. (1971) *Biochem. J. 125*, 189-201.

Morris, H. R., Dickinson, R. J., & Willians, D. H. (1973) *Biochem. Biophys. Res. Commun. 51*, 247-255.

Parente, A., Branno, M., Malorni, M. C., Welling, G. W., Libonati, M., & D'Alessio, G. (1976) *Biochim. Biophys. Acta 445*, 377-385.

Piccoli, R., & D'Alessio, G. (1984) *J. Biol. Chem. 259*, 693-695.

Piccoli, R., Di Donato, A., Dudkin, S., & D'Alessio, G. (1982) *FEBS Lett. 140*, 307-310.

Reindel, F., & W. (1954) *Chem. Ber. 87*, 1103-1107.

Shaked, L., Szajewski, R. P., & Whitesides, G. M. (1980) *Biochemistry 19*, 4156-4166.

Suzuki, H., Greco, L., Parente, A., Farina, B., La Montagna, R., & Leone, E. (1976) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) Vol. 5, Suppl. 2, p 93, N.B.R. Foundation, Washington, DC.

Synder, G. H., Cennerazzo, M. J., Karalis, A. J., & Field, D. (1981) *Biochemistry 20*, 6509-6519.

Szajewski, R. P., & Whitesides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011-2025.

Torchinsky, Yu. M. (1981) in *Sulfur in Proteins*, Pergamon Press, New York.

Whitesides, G. M., Lilburn, J. E., & Szajewski, R. P. (1977) *J. Org. Chem. 42*, 332-338.

Wilson, J. M., Bayer, R. J., & Hupe, D. J. (1977) *J. Am. Chem. Soc. 99*, 7922-7926.

Wilson, J. M., Wu, D., Motiv-De Grood, R., & Hupe, D. J. (1980) *J. Am. Chem. Soc. 102*, 359-363.

Studies of the Ammonia-Dependent Reaction of Beef Pancreatic Asparagine Synthetase†

Paige M. Mehlhaff, Craig A. Luehr, and Sheldon M. Schuster*

Department of Chemistry and School of Biological Sciences, The University of Nebraska, Lincoln, Nebraska 68588-0304

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ABSTRACT: We have studied the asparagine synthetase reaction with regard to the ammonia-dependent production of asparagine. Hydroxylamine was shown to be an alternate substrate for the asparagine synthetase reaction, and some of its kinetic properties were examined. The ammonia-dependent reaction was examined with regard to inhibition by asparagine. It was found that asparagine inhibition was partial competitive with respect to ammonia, regardless of the concentration of aspartate. However, when MgATP was not saturating, the inhibition by asparagine became linear competitive. These results were interpreted to be consistent with a kinetic mechanism for asparagine synthetase where ammonia is bound to the enzyme followed by MgATP causing asparagine release.

A pathway for asparagine biosynthesis was first noted in bacteria (Al-Dawody & Varner, 1961; Ravel et al., 1962). In this system, asparagine synthetase catalyzes the amidation of aspartate from free ammonia with the concurrent hydrolysis of ATP to AMP and pyrophosphate. In mammalian tissues, however, it was found that, in addition to ammonia, glutamine could serve as the nitrogen source for asparagine synthetase (Patterson & Orr, 1968; Arfin, 1967). Patterson & Orr (1968) reported a K_m for NH_4Cl of 120 mM for asparagine synthetase from the Novikoff hepatoma. Since the activity of the ammonia-dependent reaction increased with pH, the substrate was considered to be the free ammonia species as opposed to the ammonium ion. In the glutamine-dependent reaction, the optimum pH range was found to be from 6.6 to 8.0.

The formation of β-aspartylhydroxamate by asparagine synthetase has been reported when ammonia or glutamine is

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