limiting current collection of iodine, mimics the anodic photocurrent, with ratio corresponding to that found for the collection of these species in the dark current on the iodide wave.

These results demonstrate that C_{60} films have distinct n-type semiconductor character. Although the photovoltage and quantum efficiency are small at this stage, they have been studied only under a limited range of conditions. The effects measured here are consistent with photovoltaic junction response, but may also be modified by the photoconductive contribution.[5] We have previously noted such combined effects in junctions of a-Si:H.[13]

Work is ongoing to clarify solvent and ion effects, the extension to C_{70}, and the comparison to behavior of sublimed C_{60} films. For the latter two cases, preliminary experiments indicate photoanodic behavior at positive potentials paralleling cast C_{60} for both, while the sublimed film shows photocapacitive response at potentials negative of open circuit. We note that, although C_{60} is now known to be photodegradable[14] by UV light, no loss of photoactivity was detected in successive repetitions of voltammetry under the Ar ion laser (broadband) short term exposure in these experiments.

(13) Rosamilia, J. M.; Miller, B. *J. Electrochem. Soc.* **1985**, *132*, 349.
(14) Taylor, R.; Parsons, J. P.; Avent, A. G.; Rannard, S. P.; Dennis, T. J.; Hare, J. P.; Kroto, H. W.; Walton, D. R. M. *Nature* **1991**, *351*, 277.

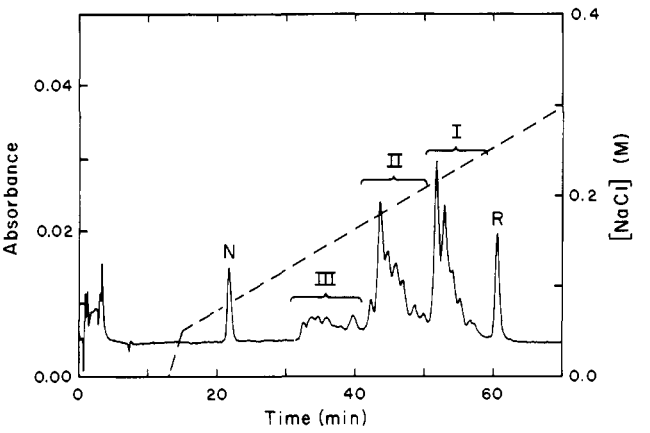

Figure 1. The solid curve represents the chromatographic separation of disulfide intermediates in the regeneration of reduced RNase A on a Bakerbond CBX column at room temperature in 25 mM Hepes, 1 mM EDTA, pH 7.0. Fully reduced RNase A (30 μM) was regenerated for 90 min at 25 °C in 100 mM Tris, 2 mM EDTA, pH 8.0. The starting concentration of DTT^{ox} was 100 mM. The regeneration process was quenched by addition of an excess of AEMTS and desalted. R is the fully reduced protein, N is the fully regenerated native protein. The Roman numerals indicate the number of intramolecular disulfide bonds of the protein in the labeled peak. The peaks at the beginning of the chromatogram pertain to buffer salts. The salt gradient used to elute the protein is represented by the dashed line.

Regeneration and Reduction of Native Bovine Pancreatic Ribonuclease A with Oxidized and Reduced Dithiothreitol

David M. Rothwarf and Harold A. Scheraga*

Baker Laboratory of Chemistry
Cornell University
Ithaca, New York 14853-1301

Received February 21, 1991

We report here the first successful regeneration of bovine pancreatic ribonuclease (RNase A) with the cyclic disulfide *trans*-4,5-dihydroxy-1,2-dithiane (DTT^{ox}) under anaerobic conditions. We also report the isolation of a native-like three-disulfide species lacking the 65–72 disulfide upon reduction of native RNase A with dithiothreitol (DTT^{red}). These results represent a significant experimental breakthrough in attempts to elucidate the regeneration pathways of RNase A and clarify a considerable amount of controversy that has arisen in the literature. The experimental techniques used to obtain these results offer the potential to resolve similar problems in other proteins and will, along with the bulk of the experimental data, be presented in greater detail elsewhere.

RNase A has been regenerated successfully with mixtures of oxidized glutathione (GSSG) and reduced glutathione (GSH),[1-4] and a number of regeneration pathways have been assigned on the basis of a kinetic analysis.[5] However, the determination of the specific disulfide-bonded intermediates that are involved in these pathways has been hampered by the complexity of the problem. Linear disulfide reagents, such as GSSG, can form stable mixed disulfides with protein thiols.[4,6] This increases the number of possible disulfide-bonded species from 763 to 7192.[7] In addition, the stability of various mixed disulfides with GSSG strongly determines which pathway is taken.[5] Because mixed disulfides with DTT^{ox} are highly unstable,[6] these problems do not exist when DTT^{ox} is used.

Previously published attempts to use DTT^{ox} in studies of the regeneration pathways of RNase A have been unsuccessful, because of an apparent inability of DTT^{ox} to regenerate RNase A.[3,8-10] A report by Pigiet and Schuster[11] indicating that DTT^{ox} can regenerate RNase A is complicated by the failure to exclude oxygen; hence, the relative contributions of air oxidation and DTT^{ox} to the regeneration process could not be evaluated.[12,13] Since DTT^{red} is a potent reducing agent,[14] the amount of DTT^{red} produced by the formation of protein disulfides inhibits the rate of regeneration. Since the thiolate anion represents the relevant reduced species responsible for the reducing power[6] of DTT^{red}, a decrease in pH will increase the oxidative strength of the redox pair significantly. While previous studies[3,8-10] have been conducted at pH 8.7, the majority of our experiments have been carried out at pH 8.0, the pH used in the previous glutathione studies of Konishi et al.[4,5] Typical results are shown in Figure 1.

An additional factor in the failure of previous workers to detect native RNase A is the lag time between the start of the oxidation process and the appearance of the native protein. This lag varies from 15–90 min and depends on the pH, temperature, and starting concentrations of DTT^{ox}, DTT^{red}, and protein. In previous regeneration studies[3,8,10] data were taken only during the first 30 min of the oxidative process. We have observed that, even under the less favorable conditions used in those earlier studies,[3,15] an apparent half-life following the lag period ($\tau_{1/2}$)[16] of 34 h can be

* Author to whom correspondence should be addressed.

(1) Hantgan, R. R.; Hammes, G. G.; Scheraga, H. A. *Biochemistry* **1974**, *13*, 3421-3431.
(2) Ahmed, A. K.; Schaffer, S. W.; Wetlaufer, D. B. *J. Biol. Chem.* **1975**, *250*, 8477-8482.
(3) Creighton, T. E. *J. Mol. Biol.* **1977**, *113*, 329-341.
(4) Konishi, Y.; Ooi, T.; Scheraga, H. A. *Biochemistry* **1981**, *20*, 3945-3955.
(5) Konishi, Y.; Ooi, T.; Scheraga, H. A. *Biochemistry* **1982**, *21*, 4734-4740.
(6) Creighton, T. E. *Methods Enzymol.* **1986**, *131*, 83-106.
(7) Appendix of ref 4.
(8) Creighton, T. E. *J. Mol. Biol.* **1979**, *129*, 411-431.
(9) Creighton, T. E.; Hillson, D. A.; Freedman, R. B. *J. Mol. Biol.* **1980**, *142*, 43-62.
(10) Wearne, S. J.; Creighton, T. E. *Proteins* **1988**, *4*, 251-261.
(11) Pigiet, V. P.; Schuster, B. J. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7643-7647.
(12) Pigiet and Schuster[11] were not studying the regeneration pathways of RNase A and made no claims as to the ability of DTT^{ox} to regenerate RNase A in the absence of oxygen.
(13) With the exception of ref 11, all regeneration studies cited here, involving thiol reagents and regeneration times longer than 30 min, were carried out under a nitrogen or an argon atmosphere. Under the conditions used in our studies, incubation of reduced protein in the absence of DTT^{ox} did not regenerate any native protein even after 48 h.
(14) Cleland, W. W. *Biochemistry* **1964**, *3*, 480-482.
(15) From ref 3, 60 mM DTT^{ox}, 30 μM RNase A, pH 8.7, 25 °C.

0002-7863/91/1513-6293$02.50/0

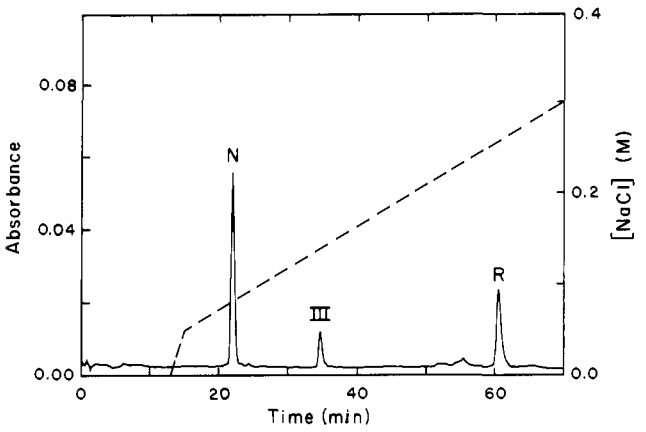

Figure 2. Chromatographic separation of disulfide intermediates in the reduction of RNase A. Native RNase A (73 μM) was reduced for 18 h at 25 °C and pH 8.0. The starting concentration of DTT^{red} was 40 mM. All other conditions are as described in Figure 1.

observed. The shortest $\tau_{1/2}$ that we have been able to observe is 128 min.[17] This compares favorably with the $\tau_{1/2}$ of 108 min obtained at the optimal ratio of oxidized and reduced glutathione under similar solution conditions.[5] Because DTT^{ox} regeneration is not limited by mixed disulfide formation, it is likely that higher ratios of DTT^{ox}/DTT^{red} will result in regeneration rates greater than those obtainable with glutathione.

The pathway for reduction of RNase A by DTT^{red} has been shown to have a native-like three-disulfide intermediate lacking the 65–72 disulfide.[18] This work has been reported by Creighton[8,10] to be nonreproducible. We have, however, been able to isolate this species in good yield, greater than 8% of the total protein, as shown in Figure 2. The primary difference between our result and previous results is the method used to block the free thiols in the protein. The blocking reagent that we used, 2-aminoethyl methanethiosulfonate (AEMTS),[19] is at least 5 orders of magnitude more reactive with thiols in solution than the reagent used in previous studies, iodoacetic acid.[20] It appears that the accessibility of the Cys-65 and Cys-72 thiols is somewhat restricted. We have found that they are not blocked by iodoacetic acid under the conditions normally employed.[3,8,10]

Conclusions[10,21] based on the previous inability of DTT^{ox} to regenerate native RNase A and the earlier failure to observe any stable intermediates upon reduction with DTT^{red} have been used to promote a specific regeneration model[3,8,22] and to attempt to discredit a more general model[5,23,24] for the regeneration pathways of RNase A. Clearly, given the data reported here, such contentions are unwarranted.

Acknowledgment. This work was supported by Grant No. GM-14312 from the National Institute of General Medical Sciences of the National Institutes of Health. Support was also received from the Cornell Biotechnology Center and from the National Foundation for Cancer Research. D.M.R. was an NIH predoctoral trainee.

Registry No. DTT^{ox}, 14193-38-5; DTT^{red}, 3483-12-3; RNase A, 9001-99-4.

(16) $\tau_{1/2}$ is determined from the average regeneration rate during the 2-h period corresponding to 1–3 h following the initiation of the oxidative process. The appearance of native protein as measured by chromatographic peak areas corresponds quantitatively to the regain of native activity.
(17) Conditions were 100 mM DTT^{ox}, 3 μM RNase A, pH 8.0, 25 °C.
(18) Sperling, R.; Burstein, Y.; Steinberg, I. Z. *Biochemistry* **1969**, *8*, 3810–3820.
(19) Bruice, T. W.; Kenyon, G. L. *J. Protein Chem.* **1982**, *1*, 47–58.
(20) Based on stopped-flow data with a mixture of GSH and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent).
(21) Creighton, T. E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5082–5086.
(22) Creighton, T. E. *Prog. Biophys. Mol. Biol.* **1978**, *33*, 231–297.
(23) Konishi, Y.; Ooi, T.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5734–5738.
(24) Scheraga, H. A.; Konishi, Y.; Ooi, T. *Adv. Biophys.* **1984**, *18*, 21–41.

Detection of Noncovalent Receptor–Ligand Complexes by Mass Spectrometry

Bruce Ganem*

Department of Chemistry, Baker Laboratory
Cornell University, Ithaca, New York 14853

Yu-Tsyr Li and Jack D. Henion*

Drug Testing and Toxicology
New York State College of Veterinary Medicine
Cornell University, 925 Warren Drive
Ithaca, New York 14850

Received April 24, 1991

Few methods are known for detecting and identifying enzyme–substrate, receptor–ligand, and antibody–antigen complexes, whose weak noncovalent interactions constitute the essential basis of molecular recognition in the biological world. With newer ionization techniques, mass spectrometry (MS) can be applied to problems of biological interest;[1] however, efforts to date have focused on sequencing carbohydrates,[2] oligonucleotides,[3] peptides, and proteins[4] and detecting other macromolecules.[5]

The newly developed technique of ion-spray (pneumatically assisted electrospray) MS forms gas-phase macromolecular ions directly from solution at atmospheric pressure via protonation and ion evaporation.[6,7] In contrast to electrospray, ion-spray MS can be performed in water without cosolvent, which is ideal for most biological systems. Multiple charging produces a family of molecular ions and dramatically reduces the mass-to-charge ratio, so that even quadrupole mass spectrometers having a typical mass range of 1000–2000 daltons (Da) can determine high MW species with unit mass resolution.

Since ionization of the targeted molecular species occurs under very mild conditions, fragmentation is usually not observed in ion-spray MS experiments, thus suggesting that noncovalent molecular association complexes might be detectable under conditions of real-time[8] reaction monitoring. Here we describe the first successful application of such a technique to a problem of considerable contemporary interest. The method should prove useful in probing a wide variety of macromolecular host–guest interactions.

The macrolides FK506 **1** (MW 804 Da, Figure 1) and rapamycin **2** (RM, MW 913 Da) are promising new immunosuppressive agents with approximately 100-fold better activity than the widely used immunosuppressant cyclosporin A.[9] Both **1** and **2** inhibit T-cell activation in a complex series of events triggered by binding of the drugs to their naturally occurring cytoplasmic receptor FKBP, a member of the immunophilin family of im-

* Address correspondence to either of these authors.

(1) (a) McLafferty, F. W. *Acc. Chem. Res.* **1980**, *13*, 33. (b) Grant, E. R.; Cooks, R. G. *Science* **1990**, *250*, 61. (c) Hodgson, J. *Bio/Technology* **1990**, *8*, 959.
(2) (a) Dell, A. *Adv. Carbohydr. Chem. Biochem.* **1987**, *45*, 19. (b) Reinhold, V. N.; Carr, S. A.; Green, B. N.; Petitou, M.; Choay, J.; Sinay, P. *Carbohydr. Res.* **1987**, *161*, 305.
(3) Laramee', J. A.; Arbogast, B.; Deinzer, M. L. *Anal. Chem.* **1989**, *61*, 2154.
(4) (a) McLafferty, F. W.; Cody, R. B., Jr.; Amster, J. *Proc. Natl Acad. Sci. U.S.A.* **1985**, *82*, 6367. (b) *Mass Spectrometry in Biomedical Research*; Gaskell, S. J., Ed.; Wiley: New York, 1986. (c) *The Analysis of Peptides and Proteins by Mass Spectometry*; McNeal, C. J., Ed.; Wiley: New York, 1988. (d) Biemann, K.; Scoble, H. A. *Science* **1987**, *237*, 992. (e) Orlando, R.; Murphy, C.; Fenselau, C.; Hansen, G.; Cotter, R. J. *Anal. Chem.* **1990**, *62*, 125.
(5) Biemann, K. *Biomed. Mass. Spectrom.* **1988**, *16*, 99 and references cited therein.
(6) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.
(7) (a) Bruins, A. P.; Covey, T. R.; Henion, J. D. *Anal. Chem.* **1987**, *59*, 2642. (b) Covey, T. R.; Bonner, R. F.; Shushan, B. I.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249.
(8) Lee, E. D.; Mück, W.; Covey, T. R.; Henion, J. D. *J. Am. Chem. Soc.* **1989**, *111*, 4600.
(9) (a) Starzl, T.; Fung, J.; Venkataramman, R.; Todo, S.; Demetris, A. J.; Jain, A. *The Lancet* **1989**, 1000. (b) Thomson, A. W. *Immunol. Today* **1989**, *10*, 6.

0002-7863/91/1513-6294$02.50/0