

*Proceedings of the*  
NATIONAL ACADEMY OF SCIENCES

---

Volume 47 · Number 9 · September 15, 1961

THE KINETICS OF FORMATION OF NATIVE RIBONUCLEASE  
DURING OXIDATION OF THE REDUCED POLYPEPTIDE CHAIN

BY C. B. ANFINSEN, E. HABER,\* M. SELA,† AND F. H. WHITE, JR.

LABORATORY OF CELLULAR PHYSIOLOGY AND METABOLISM, NATIONAL HEART INSTITUTE,  
NATIONAL INSTITUTES OF HEALTH

*Communicated by John T. Edsall, July 31, 1961*

Bovine pancreatic ribonuclease is completely reduced by treatment with mercaptoethanol in 8 *M* urea to yield a randomly coiled polypeptide chain containing eight cysteine residues.<sup>1-3</sup> Under optimal conditions of polypeptide concentration and pH, essentially complete reformation of the disulfide bonds of the native enzyme occurs in the presence of molecular oxygen.<sup>2, 3</sup> From chemical and physical studies of the reformed enzyme, it may be concluded that the information for the correct pairing of half-cystine residues in disulfide linkage, and for the assumption of the native secondary and tertiary structures, is contained in the amino acid sequence itself.

Preliminary to studies on the interactions involved in the refolding process, and to establish the order of chemical events during the formation of active protein, we have followed the rates of disappearance of sulfhydryl groups, and of the appearance of the spectral properties characteristic of the native enzyme and its active derivatives. The appearance of increased positive optical rotation associated with secondary structure was also studied. The results rule out the sequential formation of one active molecule after another. They suggest as a major possibility that some disulfide bonds formed during the early stages of oxidation are not identical with those of the native protein but undergo rearrangement to yield the native configuration.

*Materials and Methods.*—Bovine pancreatic ribonuclease (RNase) was purchased from the Sigma Chemical Company, St. Louis, Missouri. Test samples of the lot employed in these studies (#R60B-204, "chromatographic grade") were subjected, before use, to chromatography<sup>4</sup> on the cation exchanger, carboxymethylcellulose<sup>5</sup> (Brown Paper Company, Lot 1069; 0.6 meq/gm) and on IRC-50,<sup>6</sup> and were found to consist almost entirely of the major "A" peak with a smaller peak (approximately 5% of the major peak) running in the position of the usual "B" component. Amino acid analysis according to the procedure of Spackman, Stein and Moore<sup>7</sup> yielded values in good agreement with those reported for the commercial preparations that have been used for most of the structural studies on this enzyme.

Reduced RNase was prepared by treatment of the native enzyme with mercaptoethanol in 8 *M* urea followed by separation of the reduced chain from reagents on Sephadex-G25 (Pharmacia, Uppsala, Lot 6493) as described earlier.<sup>3</sup>

The procedure employed for oxidation of fully reduced RNase consisted of adjustment of the concentration of the solution of reduced protein in 0.1 *M* acetic acid that emerged from the

Sephadex-G25 columns with water to the desired protein concentration, followed by adjustment to pH 8.2 with tris (hydroxymethyl) aminomethane (tris)-acetate buffer. Three large-scale oxidations were carried out, two at 2.0 mg/ml and one at 0.1 mg/ml. Oxidation in two experiments was allowed to proceed at room temperature (23–24°C) in open beakers without stirring. In a third experiment (at 2.0 mg/ml), slow stirring was employed, without detectable differences in results except for a slight increase in the amount of turbidity produced. The soluble protein concentration, after as long as 24 hr, was still 95% or more of the original concentration. During oxidation, and particularly frequently during the early stages, samples were withdrawn, diluted to the desired concentrations with 0.1M acetate buffer, pH 5.0, and assayed<sup>8</sup> for enzyme activity against ribonucleic acid prepared according to Crestfield, Smith, and Allen,<sup>9</sup> and against uridylic-2',3'-cyclic phosphate (barium salt, Schwarz BioResearch). The latter assays were carried out with the Cary Recording ultraviolet spectrophotometer as previously described.<sup>2, 10</sup> Each analysis was compared with simultaneous controls run on native RNase solutions.

The Cary spectrophotometer was also employed for the serial determination of the absorption spectra of the solutions, with the speed of reading adjusted to yield curves that permitted accurate estimation of the differences in absorbancy at various wavelengths. Protein concentrations were estimated from the same spectra at 275 m $\mu$  where RNase and reduced RNase have the same molar extinction ( $E = 9,200$ ).

Optical rotatory measurements were made with a Rudolph precision ultraviolet polarimeter, model 80, equipped with the Rudolph photometric polarimeter attachment and an oscillating polarizer prism. The measurements were carried out at a wavelength of 366 m $\mu$ , using a mercury lamp as the light source. At this wavelength, the changes in specific optical rotation were sufficiently large to permit reasonable accuracy with 20 cm polarimetric tubes even with relatively dilute solutions of protein (2 mg/ml). The specific optical rotation for native RNase at this wavelength was  $-288^\circ$ , and for fully reduced RNase,  $-372^\circ$ . In one experiment, where oxidation was carried out on a solution of reduced RNase at a concentration of 0.1 mg/ml, samples of the reoxidation mixture were taken at various times, acidified with acetic acid to approximately pH 4, and lyophilized. The resulting material, when dissolved in water at a concentration of 2 mg/ml, gave optical rotations in excellent agreement with corresponding samples taken at similar times of oxidation in other experiments at higher protein concentrations. Most samples taken during the latter stages of oxidation were somewhat turbid and required brief centrifugation at approximately 15,000 *g* before measurement.

The extent of reduction and the kinetics of SH disappearance during oxidation were determined both by titration with *p*-chloromercuribenzoate<sup>11</sup> and by reaction with 1-C<sub>14</sub>-iodoacetic acid of known specific radioactivity followed by determination of radioactivity on the alkylated protein.<sup>3</sup> Aliquots of the protein solution (approximately 2 mg protein in 1.0 ml were added to 1.0 ml 1.0 M tris-acetate buffer, pH 8.5 containing 10  $\mu$ moles C<sub>14</sub> iodoacetic acid (0.2  $\mu$ curie) and the alkylation was allowed to proceed for 10 min. After this time, excess iodoacetic acid was immobilized by the addition of an excess (5  $\mu$ l) of mercaptoethanol. After reaction for 15 min, the alkylated protein was separated from the various reagents by passage of the sample through a (1  $\times$  40) cm column of Sephadex G25 in 0.1 M acetic acid, and the radioactivity was subsequently counted in the Packard Scintillation counter in the presence of a water-miscible phosphor.<sup>12</sup> Protein concentration in the samples counted was calculated from absorbancy measurements at 275 m $\mu$ .

**Results.**—The data in Figure 1 summarize the changes in the sulfhydryl group content, specific optical rotation and enzyme activity during oxidation of the SH groups of fully reduced RNase under the conditions presented in the experimental section. Final values obtained, expressed as percentages of the differences between fully reduced RNase and native RNase, are shown at the right of the figure. These values indicate essentially complete restoration of the native properties. A final value for the activity of the oxidation mixture toward the synthetic substrate is not given since this value was quite variable (generally 60–70% of the native level) due, possibly, to inhibition by components of the solution. This possibility has not been investigated as part of the present study. It has been found, however,

that on chromatography of samples of reformed RNase, the major component is identical to that present in commercial samples of the enzyme and possesses completely normal activity toward both RNA and synthetic substrates.<sup>2</sup>

The most striking phenomenon observed in these studies is the marked lag phase before enzymatic activity appears, during which period the sulfhydryl titer and the specific optical rotation change along a curve similar to that of a first-order reaction. The presence of this lag phase (various explanations for which are offered in the discussion) immediately rules out the possibility that disulfide bonds are formed in such a way that detectable amounts of complete, native molecules are produced in a one-by-one fashion.

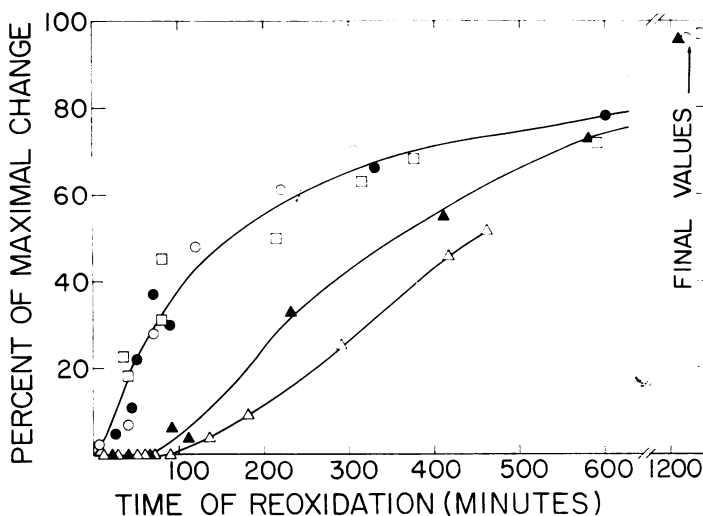


FIG. 1.—Changes, during the oxidation of reduced ribonuclease, in SH groups as followed by titration with *p*-chloromercuribenzoate (●) and by reaction with radioactive iodoacetate (O), in optical rotation (□), and in enzymatic activity as measured against ribonucleic acid (▲) and against uridylic-2,3'-cyclic phosphate (Δ).

Changes in spectral properties during oxidation (from one typical experiment) are summarized in Table 1 and conform, in general, to earlier observations on the relationship between activity and spectral properties. Native RNase shows an absorption maximum at  $277.5\text{ m}\mu$ , which is also shown by such active derivatives<sup>13, 14</sup> as RNase-S, carboxypeptidase-treated RNase and RNase which has been modified by addition of polyalanine side chains of considerable length on available amino groups.<sup>15</sup> On the other hand, inactive derivatives still possessing intact SS bonds (pepsin-inactivated RNase, methylated RNase, etc.) have shown a maximum at  $276\text{ m}\mu$ .<sup>13, 14</sup> Both oxidized and reduced RNase show still another absorption maximum, at  $275\text{ m}\mu$ , which appears to be characteristic of the chain devoid of disulfide cross-links.

In the present studies (see Table 1) the position of the maximum changes from  $275\text{ m}\mu$  to  $276\text{ m}\mu$  during the lag period prior to the appearance of enzyme activity, and then increases roughly in parallel with the appearance of enzyme activity to a

TABLE 1  
SPECTRAL CHANGES

Time, min	Wavelength of maximum absorption, $m\mu$	Molar extinction, $E_{287} m\mu$
0	275.0	3600
72	275.4	4600
177	276.2	5080
296	276.2	5470
453	276.4	5900
800	276.8	5700
Native RNase	277.5	6360

final value of 277.5  $m\mu$ . The presence of a maximum at 276  $m\mu$  is, incidentally, also characteristic of the randomly cross-linked derivative obtained during oxidation in urea, guanidine, and other disorienting reagents<sup>15</sup> and may be characteristic of tyrosine interactions of a nonspecific sort, occurring when the *specific* interaction of the phenolic hydroxyl groups is prevented.

The reaction was also followed at 287  $m\mu$ , as this is the peak of the difference spectrum between RNase and inactive RNase derivatives. Although the data are not included in Figure 1, a direct correlation becomes evident between change in activity and change in molar extinction at 287  $m\mu$  when these parameters are plotted as functions of the time of oxidation, assuming as final and initial values the absorptions at 287  $m\mu$  of native RNase and of the inactive form of the enzyme with "shifted spectrum," having a maximum at 276  $m\mu$ .

*Discussion.*—During the formation of native RNase from the reduced chain, sulfhydryl groups might be converted to disulfide bonds by one of two general mechanisms, the first involving the initial pairing of the *correct* half-cystine residues and the second, of random pairing with subsequent reshuffling to yield the native arrangement.

A. *Correct pairing:* (1) The process might proceed by a "one-by-one" mechanism in which each molecule of reduced RNase is rapidly oxidized to the native, active form without the accumulation of significant quantities of partial oxidation products. The formation of the first bond would, in this system, greatly increase the likelihood that a *second* bond would form in the same molecule over the probability that a *first* bond would form in another molecule. (2) The rates of formation of successive disulfide bonds might be sufficiently different that, at any time, the population of molecules would be *predominantly of one kind*; e.g., one SS bond with six SH groups, two SS bonds with four SH groups, etc. Although the correct pairing of SH groups is assumed in this mechanism, enzyme activity would first appear only after the formation of the final, fourth, bond (if all four are indeed essential). (3) A variation of mechanism No. 2 would involve rates of oxidation of the four pairs of SH groups that are *essentially the same*. This variation would lead to the formation of a mixture of partially oxidized molecules; *activity* would appear upon oxidation of the *fourth* pair or possibly earlier, should some molecules having only two or three disulfide bonds possess activity. (4) A situation might be visualized in which the *correct* arrangement of disulfide bonds has been formed but where the resulting molecules are inactive because of *incorrect secondary and tertiary structure*. The appearance of enzyme activity and "native" spectral properties would result from the rearrangement of noncovalent interactions to yield the native form.

cooperative  
zippering  
non-equivalent  
random,  
equivalent  
random,  
disulfide  
first

*B. Random pairing:* Pairing might be random in the initial stages of reaction and activity might first appear after reshuffling, through disulfide interchange, to yield the native configuration.

The results presented in this paper make it possible to rule out mechanism *A1* since only traces of enzyme activity and of the native spectral characteristics have appeared even after the disappearance of approximately half of the total SH group content of the reduced protein. Alternatives *A2* and *A3* are somewhat more difficult to rule out, particularly without a careful examination of the chemical nature of the disulfide bridges that have formed at various times by techniques similar to those used in establishing the disulfide bridges of the native enzyme.<sup>16</sup> However, on a purely kinetic basis, these mechanisms seem unlikely since, when three-fourths of the SH groups have disappeared, enzyme activity has risen to more than half of the theoretical value. Mechanisms *A2* and *A3* would require that no activity appear before three of the four final SS bonds were complete in all the molecules present (unless partially oxidized molecules with only three SS bonds reformed possess some activity).

Mechanism *A4* permits a lag in the appearance of activity even if the conditions of mechanism *A1* obtain. Nevertheless, this mechanism is somewhat improbable on the basis of the known characteristics of rearrangement of the secondary and tertiary structures of native RNase after distortion in solutions of 8 *M* urea. It has been shown, for example, that the modifications in spectral and optical rotatory properties of RNase occurring in urea solutions are entirely reversible upon dilution to lower urea concentrations or upon dialysis.<sup>14, 17</sup> Further, the time required for reversal is relatively short, and certainly only a small fraction of the time involved in the 1–2-hour lag period found in the present experiments. The rapid refolding of the urea-distorted configuration of native RNase by polyanions<sup>13</sup> (presumably including ribonucleic acid itself) yields an active enzyme, also militates against aberrant three dimensional structure as a major reason for the existence of a lag phase in activity in the present experiments on the reformation of SS bonds.

Mechanism *B* is consistent with the kinetic data presented here and becomes even more probable when other available information is taken into account. As mentioned above, oxidation of the SH groups of reduced RNase may be carried out in the presence of various agents that cause the formation of enzymatically inactive molecules, for which the typical spectrum of RNase, with a maximum at 277.5  $m\mu$ , is not obtained. When such randomly cross-linked molecules are incubated under the conditions employed for oxidations as described in the present paper, no detectable changes occur. However, the addition of SH compounds (including reduced RNase) under conditions known to favor disulfide interchange,<sup>18</sup> induces the rearrangement of the molecule to yield an active product in high yield, possessing the physical properties of native RNase.<sup>19</sup> The requirement for SH catalysis supports the idea that the inactive materials are inactive because of random pairing of half-cystine residues and that, in the case of the lag in appearance of enzyme activity in the present experiments, a similar situation obtains.

It is tentatively concluded, therefore, that oxidation of SH groups in this system occurs initially through relatively random formation of SS bonds with subsequent rearrangement taking place under the influence of disulfide interchange driven by

thermodynamic forces toward the most probable form, native ribonuclease. Some of the less likely possibilities mentioned above can only be rigorously excluded upon completion of current experiments on the nature of the pairing of half-cystine residues during the lag phase.

The authors wish to thank Mrs. Juanita Cooke for her expert assistance in many of these experiments.

\* Present address: Massachusetts General Hospital, Boston 14, Massachusetts.

† On leave of absence from The Weizmann Institute of Science, Rehovoth, Israel.

- <sup>1</sup> Sela, M., F. H. White, Jr., and C. B. Anfinsen, *Biochim. et Biophys. Acta*, **31**, 417 (1959).
- <sup>2</sup> White, F. H., Jr., *J. Biol. Chem.*, **236**, 1353 (1961).
- <sup>3</sup> Anfinsen, C. B., and E. Haber, *ibid.*, **236**, 1361 (1961).
- <sup>4</sup> Aqvist, S. E. G., and C. B. Anfinsen, *ibid.*, **234**, 1112 (1959).
- <sup>5</sup> Peterson, E. A., and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956).
- <sup>6</sup> Hirs, C. H. W., S. Moore, and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).
- <sup>7</sup> Spackman, D. H., W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
- <sup>8</sup> Anfinsen, C. B., R. R. Redfield, W. L. Choate, J. Page, and W. R. Carroll, *J. Biol. Chem.*, **207**, 201 (1954).
- <sup>9</sup> Crestfield, A. M., Smith, K. C., and F. W. Allen, *ibid.*, **216**, 185 (1956).
- <sup>10</sup> Richards, F. M., *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **29**, 315 (1955).
- <sup>11</sup> Boyer, P. D., *J. Am. Chem. Soc.*, **76**, 4331 (1954).
- <sup>12</sup> Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
- <sup>13</sup> Sela, M., and C. B. Anfinsen, *Biochim. et Biophys. Acta*, **24**, 229 (1957).
- <sup>14</sup> Sela, M., C. B. Anfinsen, and W. F. Harrington, *ibid.*, **26**, 502 (1957).
- <sup>15</sup> Haber, E., M. Sela, and C. B. Anfinsen, *Federation Proc.*, **20**, Part I, 217 (1961).
- <sup>16</sup> Experimental work is now in progress to determine the nature of the pairing of half-cystine residues at various times during the early stages of reoxidation. Preliminary results indicate that pairing is quite random and that "incorrectly" formed bonds are present.
- <sup>17</sup> Harrington, W. F., and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **30**, 21 (1956).
- <sup>18</sup> Sluyterman, L. A. A., *Biochim. et Biophys. Acta*, **48**, 429 (1961).
- <sup>19</sup> Haber, E., and C. B. Anfinsen, unpublished data.

## PHOTOSYNTHETIC PHOSPHORYLATION AND MOLECULAR OXYGEN\*

BY DANIEL I. ARNON,† M. LOSADA, F. R. WHATLEY, H. Y. TSUJIMOTO, D. O. HALL,  
AND A. A. HORTON

DEPARTMENT OF CELL PHYSIOLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated July 3, 1961

Oxygen and photosynthesis were first linked about 200 years ago when both were discovered almost simultaneously. The earliest concept of photosynthesis was that of planetary ventilation in which illuminated plants exchanged CO<sub>2</sub> or "bad air" for O<sub>2</sub> or "vital air" (see historical review<sup>1</sup>). A mechanism for this gas exchange was proposed in 1796 by Ingenhousz.<sup>2</sup> Green plants, he suggested, absorb from "carbonic acid in the sunshine, the carbon, throwing out at that time the oxygen alone, and keeping the carbon to itself as nourishment."<sup>2</sup>

For over a hundred years afterward, the view that CO<sub>2</sub> assimilation always involved a liberation of oxygen gas was so firmly entrenched that it was even extended to the dark CO<sub>2</sub> assimilation by chemosynthetic bacteria.<sup>3-5</sup> The idea