Sequence Homologies Among Bacterial and Mitochondrial Superoxide Dismutases

(sequencer analysis/protein evolution/endosymbiotic theory/copper-zinc and manganese dismutases)

HOWARD M. STEINMAN AND ROBERT L. HILL

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Communicated by Emil L. Smith, August 17, 1973

ABSTRACT Superoxide dismutase from chicken-liver mitochondria (manganese enzyme) and the two dismutases from *Escherichia coli* (manganese and iron enzymes) were analyzed through 29 cycles of automated Edman degradations. The high degree of homology among the amino-terminal sequences of these three dismutases corroborates their known similarity of structural and functional properties, and serves as further evidence for the endosymbiotic origin of mitochondria. In contrast, these three sequences exhibit no significant homology with the amino-terminal sequence of bovine-erythrocyte superoxide dismutase, which is consistent with the classification of eukaryotic copper-zinc dismutases as a family distinct from the manganese enzymes in stability and catalytic properties.

In 1969, the activity to disproportionate superoxide radicals was discovered as a catalytic property of a bovine-erythrocyte protein of previously unknown function (1-3). This enzyme, superoxide dismutase, was found to be a metalloprotein with 2 (1.8-2.0) atoms of copper, and 2 (1.7-1.9) atoms of zinc per molecule of molecular weight 33,000, and to be composed of two subunits of equal size (4, 5). Copper-zinc dismutases purified from other eukaryotic organisms are similar to the bovine-erythrocyte dismutase in molecular weight, subunit structure, amino-acid composition, copper-zinc content, and stability to the chloroform-ethanol mixtures used in purification (2, 3). Enzymes of bacterial origin represent a distinct class of superoxide dismutases which have 1-2 atoms of manganese per molecule as metal cofactor, are unstable to the chloroform-ethanol treatment, and have an amino-acid composition distinctly different from the copper-zinc dismutases (2, 3, 6-8). The molecular weight of the bacterial enzymes is about 40,000, and each contains two subunits of molecular weight 20,000. Recently two additional superoxide dismutases have been isolated, which are similar to the bacterial manganese dismutases in their stability, purification properties, and amino-acid composition. One, from chicken-liver mitochondria (8), contains 2.3 atoms of manganese per molecule, and although it is tetrameric, has a subunit molecular weight the same as the bacterial manganese-containing enzymes. The other, which contains iron (about 1 atom per molecule) rather than manganese, has been isolated from *Escherichia coli* (9), and is a dimer whose subunits are identical in size (molecular weight 19,000).

The levels of superoxide dismutase activity have been measured in a variety of aerobic, anaerobic, and aerotolerant anaerobic microorganisms (10). From the observed correla-

tions and subsequent studies (11), it was proposed that the ability of an organism to survive in an aerobic atmosphere is at least in part contingent upon the presence of the enzyme, superoxide dismutase, which serves as a defense mechanism against oxygen toxicity (2, 3, 10, 11).

The amino-terminal sequences, obtained through automated Edman degradations, are reported here for superoxide dismutases from *E. coli* (iron- and manganese-containing enzymes) and from chicken-liver mitochondria (manganese-containing enzyme). From the homologies observed among these three sequences and the partial sequence of the bovine-erythrocyte (copper-zinc) dismutase (Evans, H. J., Steinman, H. M., Naik, V. R., Abernethy, J. L. & Hill, R. L., unpublished results), interpretations are made apropos to the structural, functional, and evolutionary properties of superoxide dismutases. The sequence comparison of the chicken mitochondrial and the *E. coli* manganese dismutases provides supporting evidence for the endosymbiotic theory of mitochondrial origin (12-14).

MATERIALS AND METHODS

Purification of Superoxide Dismutases. The enzymes used for sequencer analysis were prepared by Dr. Frederick J. Yost, Jr. and Mr. Richard A. Weisiger, according to published procedures for dismutases from *E. coli* (iron and manganese enzymes, 6, 9) and from chicken-liver mitochondria (8). The bovine-erythrocyte enzyme was purified (1) by Dr. Herbert J. Evans and Mr. John L. Abernethy.

Reduction and S-Carboxymethylation. Each dismutase was desalted by dialysis against 10% (v/v) acetic acid, then against water, and finally freeze-dried. The proteins (10 mg/ml) were then reduced with 2-mercaptoethanol (0.4-0.5 M, 5.5-6.5 hr, 37°) and alkylated (15 min, 25°) with iodo-[¹⁴C₂]acetic acid (New England Nuclear Corp., diluted with recrystallized unlabeled iodoacetic acid to 0.04-0.08 mCi/mmol) in 6 M guanidine hydrochloride (pH 8.3-8.6 in 1 M Tris·HCl), by a published protocol (15). The S-carboxymethylated proteins were then desalted by dialysis against 10% acetic acid, and stored as frozen solutions until used for sequencer analysis. The bovine-erythrocyte enzyme was treated similarly, with nonradioactive iodoacetic acid.

Sequence Analysis by Automated Edman Degradations. Analyses with a Beckman model 890B Sequencer used the program strategy originally proposed for the automated Edman degradation (16), with several recently suggested modifications (17). The coupling buffer, containing Quadrol as

Abbreviation: PTH, phenylthiohydantoin.

FIG. 1. Comparative amino-terminal sequences. Boxes enclose identical residues in the sequences of the two *E. coli* and the mitochondrial dismutases. Underlined capital letters indicate identities between any one or more of these three dismutases and bovine-erythrocyte superoxide dismutase.

tertiary amine, and other sequencer reagents (phenylisothiocyanate and heptafluorobutyric acid) were obtained from Pierce Chemical Co. ("sequanal" grade). The sequencer solvents ("distilled-in-glass" grade, heptane, ethyl acetate, benzene, and 1-chlorobutane) were purchased from Burdick and Jackson Laboratories. Sequencer fractions, after conversion (17, 18), were analyzed by gas chromatography (Beckman GC 45, SP-400, 10% on Supelcoport, nitrogen as a carrier gas) which allowed unambiguous identification of most of the amino-acid phenylthiohydantoin (PTH) derivatives (19-21). In discriminating between glycine-PTH and threonine-PTH, and between threonine-PTH and proline-PTH, gas chromatography assignments were confirmed by silica gel thin layer chromatography (solvent XM, 22). Spot tests for identification of arginine-PTH (23) and histidine-PTH (24) were performed upon the aqueous phases from the conversions after samples were dried under reduced pressure and after the residue was dissolved in methanol. Fractions identified as "serine" by gas chromatography were assigned as either serine-PTH or *S*-[¹⁴C₂]carboxymethyl cysteine-PTH by liquid scintillation counting (Packard Liquid Scintillation Spectrometer, model 577). Authentic PTH-amino acids (Pierce Chemical Co.) were used to calibrate the gas chromatograph for quantitation. The amount of protein used for sequencer analysis was calculated from the weight of the desalted, freeze-dried samples, which were subsequently reduced and carboxymethylated.

RESULTS

Sequencer Analyses of Dismutases. The two *E. coli* and the chicken-liver mitochondrial enzymes possessed free α -amino groups, permitting automated Edman degradations, whose quantitative results are presented in Table 1. Duplicate analyses of each reduced and carboxymethylated protein were qualitatively identical and quantitatively similar. Within the limitations of this methodology (17), only a single, predominant amino-acid derivative was detected at each cycle for

which an identification could be made. The average stepwise repetitive yield for the first 20 cycles ranged between 0.91 and 0.96, and between 0.86 and 0.96 for the remaining cycles reported. The amino-terminal serine of the two *E. coli* enzymes and the amino-terminal lysine of the mitochondrial dismutase are residues whose PTH derivatives are not accurately quantitated by the techniques used here (17). Therefore, the expected molar yield for these amino-terminal residues was calculated from the repetitive yield value and the yields of stable, quantifiable PTH-amino acids located near the amino terminus.

From duplicate sequencer analyses, the mol of terminal amino-acid residue per mol of subunit polypeptide chain were thus calculated: 0.73 and 0.70 mol/mol for *E. coli* (iron), 0.92 and 0.73 for *E. coli* (manganese), and 0.78 and 0.56 for the mitochondrial (manganese) dismutase. The last value for the mitochondrial enzyme was obtained for an analysis of a rather small amount of protein (1.6 mg). The average yield of amino-terminal valine on four sequencer analyses of apo sperm-whale myoglobin, a protein frequently used for sequencer standardization, was 0.78 ± 0.07 mol/mol of polypeptide chain, based upon the weight of freeze-dried protein used, uncorrected for residual salts and moisture. The values obtained for the *E. coli* and mitochondrial dismutases are thus consistent with an integral value of 1 mol of α -amino group per mol of subunit.

Comparative Sequences of Dismutases. The amino-terminal sequences determined for the two *E. coli* and the chicken-liver mitochondrial enzymes are compiled in Fig. 1, and are compared with the amino-terminal 29 residues of bovine-erythrocyte superoxide dismutase (Evans, H. J., *et al.*, unpublished results). The α -amino group of the bovine-erythrocyte enzyme is acetylated (Evans, H. J., *et al.*, unpublished results), obviating the use of sequencer analysis, and necessitating other procedures for sequence determination.

From Fig. 1, it is evident that the number of identical amino-acid residues at corresponding positions in the *E. coli* (iron and manganese) and mitochondrial (manganese) enzymes (identities enclosed by lines), vastly exceeds the identities between any one of these three dismutases and the erythrocyte (copper-zinc) enzyme (identities indicated by underlined, capital letters).

DISCUSSION

The understanding of the subunit composition, structure-function correlations, and evolutionary relationships of several families of enzymes has been enhanced considerably through amino-acid sequence analysis of representative proteins from diverse taxa (25-27). Although the primary structural data reported here are limited, being amino-terminal sequences from only three species, they do permit some inferences to be drawn about the structural, functional, and evolutionary characteristics of superoxide dismutases. At the present time, these inferences must be considered provisional, contingent upon acquisition of more complete sequence data from a larger phylogenetic sampling of dismutases.

Sequence Homologies among the Mitochondrial and Bacterial Dismutases. It is evident upon inspection that a very high degree of homology exists among the amino-terminal sequences of the two *E. coli* and the mitochondrial dismutases (Fig. 1). In the alignment shown, there are 10 loci at which the same amino-acid residues appear in all three sequences. In the sequence of the mitochondrial dismutase, 20 out of 27 residues are identical, with one or both residues at the same locus in the sequences of the two *E. coli* dismutases. In the comparison of the *E. coli* (iron) sequence with the mitochondrial and *E. coli* (manganese) sequences, this fraction is 19 out of 28 residues. When the *E. coli* (manganese) sequence is compared with the two other homologous sequences, the fraction is 19 out of 27 residues. The nonidentical residues at a specific locus in these three sequences are interconvertible by a minimum of 1 base change per RNA codon in 13 cases, and by a minimum of 2 base changes per codon in 8 cases (28).

In contrast, there is a complete absence of significant homology between the amino-terminal sequence of bovine-erythrocyte (copper-zinc) superoxide dismutase* and the sequences of the bacterial and mitochondrial dismutases (iron- or manganese-containing enzymes). Of the 29 residues in the erythrocyte sequence, only three are identical with residues at corresponding positions in one or more of the three homologous dismutases. The conversions between the non-identical isologous residues of the erythrocyte dismutase and the bacterial and mitochondrial dismutases require a minimum change of 1 base per codon in 38 instances, of 2 bases per codon in 38 instances, and of 3 bases per codon in one instance.

When computer methodology (28) was used for quantitatively assessing these sequence comparisons, the homologies among the mitochondrial and the two *E. coli* dismutases were found to be nearly as great as those between phylogenetically distinct cytochromes *c*, a protein exhibiting exceptionally high

sequence conservation throughout its evolutionary development (26, 29). Between the bovine-erythrocyte dismutase and the bacterial-mitochondrial dismutases, the sequence homologies were found to be no greater than those between completely nonhomologous proteins.

Evolutionary Relationships among the Dismutases. The iron- and manganese-containing enzymes from *E. coli* show several structural similarities; both are dimeric proteins of comparable subunit molecular weight, and their amino-acid compositions are similar (6, 9). However, it has been speculated that the iron enzyme functions primarily in the defense against external superoxide radicals, while the manganese enzyme defends against intracellular O_2^- , because the iron dismutase appears to be preferentially located in the periplasmic space, and the manganese dismutase in the cell matrix (30). The highly homologous amino-terminal sequences of the two *E. coli*

TABLE 1. *Sequencer analyses of superoxide dismutases**

Cycle	<i>E. coli</i> (Fe)		<i>E. coli</i> (Mn)		Chicken-liver mitochondrial (Mn)	
	Amino acid-PTH	Yield (nmol)	Amino acid-PTH	Yield (nmol)	Amino acid-PTH	Yield (nmol)
1	Ser	†	Ser	†	Lys	120
2	Phe	220	Tyr	300	His	†
3	Glu	310	Thr	†	Thr	†
4	Leu	240	Leu	230	Leu	150
5	Pro	300	Pro	200	Pro	160
6	Ala	360	Ser	†	Asp	130
7	Leu	250	Leu	190	Leu	160
8	Pro	240	Pro	210	Pro	110
9	Tyr	300	Tyr	200	Tyr	170
10	Ala	320	Ala	230	Asp	92
11	Lys	170	Tyr	180	Tyr	170
12	Asp	180	Asp	120	Gly	71
13	Ala	230	Ala	160	Ala	110
14	Leu	120	Leu	120	Leu	100
15	Ala	210	Glu	86	Glu	63
16	Pro	130	Pro	59	Pro	68
17	His	†	His	†	His	†
18	Ile	140	Phe	78	Ile	91
19	Ser	†	Asp	46	Ser	†
20	Ala	150	Lys	18	Ala	72
21	Glu	91	Gln	†	Glu	46
22	?	§	Thr	†	Ile	69
23	Ile	130	Met	30	Met	44
24	Glu	81	Glu	14	Gln	†
25	Tyr	120	Leu	50	Leu	58
26	His	†	?	§	His	†
27	Tyr	100	His	†	?	§
28	Gly	54	?	§	?	§
29	Lys	30	Lys	5	Lys	8

* The amounts of reduced and carboxymethylated proteins used were: 10.4 mg of *E. coli* (Fe), 7.5 mg of *E. coli* (Mn), and 5.1 mg of chicken-liver mitochondrial dismutase (Mn). The nanomolar yields were determined by gas chromatography.

† These PTH-amino acids are determined only qualitatively by gas chromatography.

‡ The Pauli spot test was used to identify histidine-PTH.

§ No identification could be made by either gas chromatography or spot tests.

* About 90% of the primary structure of bovine-erythrocyte superoxide dismutase is known, with the undetermined region representing a continuous sequence of about 15 unordered amino-acid residues, beginning about 55 residues from the amino terminus (Evans, H. J., *et al.*, unpublished results).

enzymes leave little doubt that they arose from a common ancestral dismutase. The development of their present intracellular locations and specialized functions may have occurred through duplication of the gene of the common precursor, followed by divergent evolution, as is believed to be the case with the development of the α and β chains of hemoglobin from a common ancestor (26, 27).

The stability, cyanide sensitivity, and amino-acid composition of the copper- and zinc-containing bovine-erythrocyte dismutase, and of other copper-zinc eukaryotic dismutases, characterize them as a class of enzymes that is distinct from the bacterial-mitochondrial class of dismutases (3, 6-9). Indeed, the amino-terminal sequence of the erythrocyte protein displays no significant homology to the sequences of dismutases from the bacterial-mitochondrial class, which suggests that the two classes of superoxide dismutases arose by parallel evolution, evolving independently, from separate ancestors (26, 27). The different classes of ribonucleases and the different types of lysozymes are examples of parallel evolution of protein classes, in which the members of the separate parallel branches are distinguished by different catalytic properties, as is the case with the dismutases, as well as by different primary structures (26, 27).

Although the amino-terminal sequences of the bacterial-mitochondrial and the copper-zinc enzymes are completely unrelated, significant homologies between the two could conceivably be found in unsequenced portions of their primary structures, which would suggest that the two dismutase classes arose by divergent evolution from a common ancestor. The example of divergent evolution of α -lactalbumins and lysozymes (31-33) is particularly relevant here. Relatedness or the lack of relatedness cannot be deduced from comparisons of short sections of the two sequences, as the degree of homology between α -lactalbumins and lysozymes is quite variable, being extremely high in some regions of their sequences, and barely detectable in other regions (33).

Dismutase Homologies and the Endosymbiotic Origin of Mitochondria. The endosymbiotic theory proposes that the present-day mitochondria of eukaryotic organisms have evolved from bacteria, which existed as intracellular symbionts in primitive eukaryotes (34-36). As the atmosphere of the primitive earth changed from anaerobic to aerobic, these symbionts were to have been acquired as a means of confronting selection pressures that necessitated oxidative metabolic pathways. This theory has been supported by the morphological and biochemical similarities between mitochondria and bacteria (36), by the taxonomic advantages consequent to the theory (37), and by the existence of RNA, DNA, and protein synthesis in mitochondria (34). Criticism of endosymbiosis has been based upon fossil evidence dating the emergence of eukaryotes, and upon biochemical evidence suggesting that the primitive eukaryotic cytoplasm was already adapted to the use of oxygen (12, 38). It is not the intent of this report to evaluate the arguments for and against the theory (13, 14), but rather to present the amino-terminal sequences of bacterial and mitochondrial superoxide dismutases as direct, chemical evidence, in support of endosymbiosis. The ultimate acceptance or rejection of the endosymbiotic theory will result only from evidence accumulated from the many different scientific disciplines associated with its content, assumptions, and predictions.

The amino-terminal sequence of chicken-liver mitochondrial

(manganese) superoxide dismutase is highly homologous to the sequences of the two dismutases (manganese and iron) of *E. coli* (Fig. 1), in spite of the phylogenetic distance between chickens and bacteria. In contrast, there is no detectable homology between the sequence of chicken-mitochondrial and bovine-erythrocyte (copper-zinc) superoxide dismutases, although the taxonomic distance between chickens and oxen is much less. The most reasonable conclusion consistent with these comparisons is that the structural genes for the mitochondrial and the *E. coli* dismutases have evolved from a common ancestor.

The significance of this sequence homology to the endosymbiotic theory of mitochondrial origin would seem to be obvious. The common ancestral gene predicted by the sequence homologies may have resided in the genome of the proposed symbiotic bacterium inhabiting the primitive eukaryotic cell. This correlation between endosymbiosis and dismutase evolution cogently supports the endosymbiotic theory. However, arguments in opposition to the theory cannot be refuted by these sequence homologies alone.

The extensive homology observed between the taxonomically distant chicken-mitochondrial and *E. coli* enzymes may be the consequence of an unusually low rate of evolutionary change within the bacterial-mitochondrial class of dismutases. For reasons of their cellular location and biological function, these dismutases may then be akin to the cytochromes *c* (26, 29) and histone IV (26, 39), which show extensive sequence conservation throughout their evolutionary development. It is possible that the sequences of phylogenetically distant dismutases from the copper-zinc class will also show a high degree of homology among themselves, while being markedly different from the bacterial-mitochondrial sequences. Investigations of the primary structures of additional superoxide dismutases, from these two classes and from diverse species, will answer these intriguing questions of dismutase evolution.

Subunit Structure of Superoxide Dismutases. Superoxide dismutases from *E. coli* (iron- and manganese-containing enzymes) are dimeric (6, 9), while the chicken-liver mitochondrial (manganese) enzyme is tetrameric (8). In each instance, the subunit polypeptide chains constituting the particular multimer have essentially identical molecular weights. Through sequencer analysis, a single, unique amino-terminal sequence and an integral yield of the amino-terminal residue have been identified for these three dismutases (Table 1), which are consistent with the chemical identity of the subunits within each. In sequence studies of the bovine-erythrocyte enzyme (Evans, H. J., *et al.*, unpublished results), the recoveries of peptides are also consistent with chemically identical subunits.* In contrast to the apparent homogeneous subunit structures of these four dismutases, human-erythrocyte superoxide dismutase has been reported to be a dimer of two chemically nonidentical polypeptide chains (40, 41).

Sequence Homologies and Dismutase Function. The many sections of sequence that are identical in the mitochondrial and the *E. coli* dismutases (Fig. 1) might be proposed as regions essential for dismutase function, since they are invariant in phylogenetically distant species (26, 27). However, it is evident that a completely nonhomologous amino-terminal sequence in the bovine-erythrocyte enzyme is also compatible with dismutase catalytic activity. A more complete sequence analysis of superoxide dismutases of the bacterial-mitochon-

drial and the copper-zinc classes is required before the functional importance of invariant amino-acid residues can be ascertained.

We greatly appreciate the generosity and interest of Dr. Irwin Fridovich, Department of Biochemistry, Duke University, and members of his laboratory, Dr. Frederick Yost, Jr., Dr. Henry Forman, Mr. Richard Weisiger, and Mr. Bill Lippitt. We thank Dr. Charles R. Cantor, Department of Chemistry, Columbia University, for providing the original form of the computer program used for sequence comparisons, Mr. Mark Polokoff for performing the gas chromatographic analyses and spot tests in the sequencer runs, and Jane Richardson for her advice about computer technology and programming. The interest and advice of Dr. Emil L. Smith, Department of Biological Chemistry, University of California, Los Angeles, is also gratefully acknowledged. This research was supported by Grant HL06400 from the National Heart and Lung Institute, National Institutes of Health.

1. McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049-6055.
2. Fridovich, I. (1972) *Accounts Chem. Res.* **5**, 321-326.
3. Fridovich, I. (1973) *Advan. Enzymol.*, in press.
4. Carrico, R. J. & Deutsch, H. F. (1970) *J. Biol. Chem.* **245**, 723-727.
5. Keele, B. B., Jr., McCord, J. M. & Fridovich, I. (1971) *J. Biol. Chem.* **246**, 2875-2880.
6. Keele, B. B., Jr., McCord, J. M. & Fridovich, I. (1970) *J. Biol. Chem.* **245**, 6176-6181.
7. Vance, P. G., Keele, B. B., Jr. & Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 4782-4786.
8. Weisiger, R. A. & Fridovich, I. (1973) *J. Biol. Chem.* **248**, 3582-3592.
9. Yost, F. J., Jr. & Fridovich, I. (1973) *J. Biol. Chem.* **248**, 4905-4908.
10. McCord, J. M., Keele, B. B., Jr. & Fridovich, I. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1024-1027.
11. Gregory, E. M. & Fridovich, I. (1973) *J. Bacteriol.* **114**, 1193-1197.
12. Raff, R. A. & Mahler, H. A. (1972) *Science* **177**, 575-582.
13. Uzzell, T. & Spolsky, C. (1973) *Science* **180**, 516-517.
14. Raff, R. A. & Mahler, H. A. (1973) *Science* **180**, 517.
15. Hirs, C. H. W. (1967) in *Methods in Enzymology*, ed. Hirs, C. H. W. (Academic Press, New York), Vol. XI, pp. 199-203.
16. Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80-91.
17. Hermodson, M. A., Ericsson, L. H., Titani, L., Neurath, H. & Walsh, K. A. (1972) *Biochemistry* **11**, 4493-4502.
18. Ilse, D. & Edman, P. (1963) *Aust. J. Chem.* **16**, 411-416.
19. Pisano, J. J. & Bronzert, T. J. (1969) *J. Biol. Chem.* **244**, 5597-5607.
20. Pisano, J. J., Bronzert, T. J. & Brewer, H. B., Jr. (1972) *Anal. Biochem.* **45**, 43-59.
21. Niall, H. D. (1973) in *Methods in Enzymology*, eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York), Vol. XXVII, in press.
22. Inagami, T. & Murakami, K. (1972) *Anal. Biochem.* **47**, 501-504.
23. Yamada, S. & Itano, H. A. (1966) *Biochim. Biophys. Acta* **130**, 538-540.
24. Smith, I. (1960) *Chromatographic and Electrophoretic Techniques, Volume I. Chromatography* (William Heinemann Medical Books, London), p. 296.
25. Dayhoff, M. O., ed. (1972) *Atlas of Protein Sequence and Structure 1972, Volume 5* (National Biomedical Research Foundation, Silver Spring, Md.).
26. Smith, E. L. (1970) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), Vol. I, 3rd ed. pp. 267-339.
27. Dixon, G. H. (1966) in *Essays in Biochemistry*, eds. Campbell, P. N. & Greville, G. D. (Academic Press, London), Vol. 2, pp. 147-204.
28. Jukes, T. H. & Cantor, C. R. (1969) in *Mammalian Protein Metabolism*, ed. Munro, H. N. (Academic Press, New York), Vol. III, pp. 21-132.
29. Margoliash, E. & Smith, E. L. (1965) in *Evolving Genes and Proteins*, eds. Bryson, V. & Vogel, H. J. (Academic Press, New York), pp. 221-242.
30. Gregory, E. M., Yost, F. J., Jr. & Fridovich, I. (1973) *J. Bacteriol.* **115**, 987-991.
31. Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C. & Hill, R. L. (1969) *J. Mol. Biol.* **42**, 65-86.
32. Vanaman, T. C., Brew, K. & Hill, R. L. (1970) *J. Biol. Chem.* **245**, 4583-4590.
33. Brew, K., Steinman, H. M. & Hill, R. L. (1973) *J. Biol. Chem.* **248**, 4739-4742.
34. Sagan, L. (1967) *J. Theor. Biol.* **14**, 225-274.
35. Stanier, R. Y. (1970) in *Organization and Control in Prokaryotic and Eucaryotic Cells, Twentieth Symposium of the Society of General Microbiology*, eds. Charles, H. P. & Knight, B. C. J. G. (University Press, Cambridge), pp. 1-38.
36. Raven, P. H. (1970) *Science* **169**, 641-646.
37. Margulis, L. (1968) *Science* **161**, 1020-1022.
38. Cohen, S. S. (1970) *Amer. Sci.* **58**, 281-289.
39. DeLange, R. L., Fambrough, D. M., Smith, E. L. & Bonner, J. (1969) *J. Biol. Chem.* **244**, 5669-5679.
40. Carrico, R. J. & Deutsch, H. F. (1969) *J. Biol. Chem.* **244**, 6087-6093.
41. Hartz, J. W. & Deutsch, H. F. (1972) *J. Biol. Chem.* **247**, 7043-7050.