September 1985

Final Report

[Grant Number: NCC 2-85]

# Structure and Function of Isozymes: Evolutionary Aspects and Role of Oxygen in Eucaryotic Organisms


by

Dr. Tanjore Satyanarayana

Carbon, hydrogen, oxygen and nitrogen are the four principal elements that contribute to living matter. In catalysis and energy exchange reactions iron and phosphorous are of critical importance. Each of these elements is a common constituent of the earth and presumably formed during the formation of the solar system. Carbon monoxide, carbon dioxide, water, methane, ammonia and hydrogen and oxygen in molecular form ($H_2$ and $O_2$) are common molecules formed by the important elements. All of these, except for oxygen have been considered to be the constituents of the primitive atmosphere. Oxygen is not only one of the most abundant elements on the earth, but it is also one of the most important elements for life. Such being the case, it has been the subject of intensive investigation among a number of physiologists and biochemists since the study of biological oxidation processes was initiated by Lavoister about 200 years ago. In terms of composition, the feature of the atmosphere that most distinguishes earth from other planets is the presence of abudant amounts of oxygen. If one understands what determined the oxygen content of the atmosphere today, then that will most likely unravel the history of the abundance of atmospheric oxygen.

The upper atmosphere of the earth is as dry as the Martian atmosphere, and it has been proposed by Urey that the oxygen on earth was produced by the photo dissociation of water. Further studies by Urey have revealed that there would be shielding of the main body of water vapor in the troposhere, thus resulting in an auto-regulated level of oxygen produced in a primitive atmosphere, a condition that is remarkable stable.

Berkner and Marshall calculated this level to be about 1/1000th or less of the oxygen of the present day atmosphere.

It is usually thought that the earth was once completely anaerobic, containing methane and, possibly, more complicated organic compounds. The first forms of life may have been similar to present day anaerobic bacteria such as clostridium.

Evolution of the photosynthetic cleavage of water to oxygen was doubtless a major event with far-reaching consequences. Biologists generally believe that as oxygen accumulated in the earth's atmosphere, the obligate anaerobes became limited to strictly anaerobic environments. In the meantime, new classes of bacteria appeared with microorganisms for detoxifying oxygen and for using oxygen to obtain energy.

The relationship between prokaryotes and eukaryotes, if any, has been a topic of much speculation. Eukaryotic cells contain a variety of membraneous organelles, at least some of which are probably the descendants of prokaryotic ancestors. The question of the time of origin of the eukaryotes is of interest in relation to NASA's program on the origin and early evolution of life. With only a few exceptions eukaryotes are oxygen-utilizing organisms. One question we would like to examine is whether eukaryotes or eukaryotic biochemical processes requiring oxygen, could have arisen quite early in evolution and utilized the small quantities of photocatalytically produced oxygen which are thought to have been present on the earth prior to the evolution of massive amounts of photosynthetically-produced oxygen. An insight into the time of origin of the first eukaryotes may be

obtained by studying the cellular processes affected by oxygen. One candidate for such a study would be the acetyl-coenzyme A (ACS) synthetase of Saccharomyces cerevisiae.

The existence in the microbial world of organisms capable of both fermentation and respiration, dependent on the oxygen level of their environments, is of importance for the understanding of the early evolution of life. Oxygen has a pronounced effect on the growth, general metabolism and lipid composition of the Eucaryote, yeast. The genus Saccharomyces has been used to study the effects of oxygen on various physiological parameters of yeast. These organisms provide several systems for study of the role of oxygen in Eukaryotes. There is, however, a great deal of uncertainty as to the precise role of oxygen in determining the morphological and biochemical changes that are seen in aerobically metabolizing yeast cells. It is well known that the synthesis of unsaturated fatty acids, sterols, ubiquinone and a number of hemoproteins is inhibited by exluding oxygen from the culture medium. This is a consequence of the direct involvement of molecular oxygen in the biosynthesis of the substances. However, other changes are known to occur in these yeast cells as a consequence of non-aerobic environment, for example, degradation of mitochondria, and, in turn, enzymes of tricarboxylic acid cycle.

Oxygen tension and glucose repression, regulate the formation of mitochondria in yeast. One of the enzymes associated with this organelle is acetyl-Coenzyme A synthetase (ACS), which is the key enzyme concerned with lipid synthesis in S. cerevisiae. Earlier studies on this enyzme have revealed the presence of two

distinct acetyl-CoA synthetases dependent on the availability of oxygen during growth of this organism. Under aerobic or oxygenic conditions, the enzyme is associated with the mitochrondrial fraction. Under non-aerobic conditions, it is associated with the microsomal fractions of the cell homogenate. Further studies, using isolated fractions revealed that the enzyme formed during the two conditions of growth differed in a number of ways. The affinity constant for acetate, was 10-fold higher and for ATP, 3-4 fold higher for the non-aerobic enzyme compared to the aerobic variety of the enzyme. Long chain acyl-Coenzyme A compounds (palmityl, stearyl and oleolyl) were found to be potent inhibitors of ACS from aerobic, but not from non-aerobic cells.

The inhibitory pattern by these long chain acyl-CoA compounds had characteristics of regulatory enzymes, with an interaction co-efficient of 3.25. This suggested that the aerobic enzyme is trimeric in structure. Short chain acyl-CoA compounds (propionyl, butyryl and valeryl) had no effect on ACS from either aerobic or non-aerobic source.

The "aerobic" ACS was purified to homogeneity. By various criteria, the molecular weight has been deduced to be around 250,000. This study confirms the enzyme to be a trimer with a subunit size around 83,000, this concurring with acyl-CoA inhibition studies.

In further experiments, some of the immunologic properties of the two ACS's were studied in detail. The antibody produced by each enzyme is immunologically specific. Antibodies prepared from homologous enzyme inhibited only the homologous enzyme

actifity but not the heterologous enzyme activity.

During these immunological studies, a partially purified non-aerobic preparation of the enzyme has been used. This was necessitated because attempts to purify this enzyme further were hindered because of the labile nature of the enzyme during purification procedures carried out at $4^{0}C$. Indeed both the enzymes were found to be unstable upon storage conditions at $4^{0}C$. However, addition of boiled extracts of aerobic, non-aerobic or "petite" strains of the parent strain, prevented such losses of activity for both the aerobic and non-aerobic ACS's. The instability of both the enzymes was shown not to be due to the interference of intracellular proteinases since specific inhibitors of these proteinases had no effect in preventing ACS from inactivation. The stabilizing factor (S.F.) present in the boiled extracts showed differential effects during further characterization. The S.F. was non-dialysable in the case of non-aerobic ACS stability while it was partially dialysable with regards to aerobic ACS. Charcoal treatment of the boiled extracts abolished the stabilizing capacity toward both the enzymes. Pronase treatment destroyed the S.F. when tested against the non-aerobic ACS but had no effect on the aerobic ACS. The S.F. was partially purified on G-100 Sephadex columns, and during such studies the S.F. cochromatographed with cytochrome C. indicating the molecular weight to be around 13,000. Further, the S.F. showed a single absorption maximum at 260 nm. These studies indicated the S.F. composition may be nucleopeptidic in nature.

While several nucleotides afforded protection to aerobic ACS, only adenine derivatives, ATP and ADP afforded complete protection at physiological concentrations. However, none of these nucleotides showed any capacity to protect non-aerobic ACS. Digestion of S.F. with either ribonuclease T, or deoxyribonuclease did not eliminate the protective capacity against either ACS. The ratio of the absorbance at 280 to 260 nm of S.F. coincided only with Poly G absorption characteristics. However, synthetic Poly G afforded protection only to aerobic ACS but inactivated non-aerobic ACS. This property of Poly G could be abolished by digestion with ribonuclease T. Since the digestion studies with the ribonuclease T, showed differential effects compared to S.F., the nucleotide portion of the S.F. may not be guanine nucleotide derivative. This is in complete accord since only adenine nucleotides were effective as indicated above.

Further prolification of the nucleopeptide on ion-exchange columns completely dissociated the 260 nm absorbing material from the peptide moiety. The nucleotide was identified as ATP both by ATPase digestion studies and its substitution in the in vitro assays replacing ATP. The peptide moiety was purified to homogeneity as revealed by several physical studies. Its apparent molecular weight was estimated to be around 13,000 500 by gel permeation chromatography. The stabilizing property of the peptide was abolished completely by digestion with chymotoypsin only but not by any other proteolytic enzyme.

Using the purified peptide during the purification of

the non-aerobic Acs, a 1300 fold purification was achieved. On disc gel electrophoresis, the material revealed only two bands. Whether these two bands corresponded to the subunits of the same enzyme could not be ascertained due to lack of sufficient material.

Finally the existence of two isozymes was confirmed by mixing experiments. Both the purified enzymes, when mixed, could be separated as Bio gel - columns as well as disc gel electrophoresis. While [two project has contributed immersely towards the understanding of the regulation and structure aspects of the isozymes], the exact role of oxygen and the evolutionary relationships to categorize the enzymes into convergent or divergent evolutionary pattern cannot be ascertained at this stage of the project due to the difficulty in obtaining sufficient amounts of proteins to do the detailed structural analysis.

1. Inhibition of Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae by Long-Chain Acyl- Coenzyme A Compounds. T. Satyanarayana and Harold P. Klein. Proceedings of the IVth International Fermentation Symposium on Yeasts. Kyoto, Japan, April 1972.

2. Inhibition of Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae by Long-Chain Acyl Coenzyme A Compounds. T. Satyanarayana and Harold P. Klein. American Chemical Society 164th National Meeting, August 27-Sept 1, New York, (1972).

3. Isolation and Crystallization of Acetyl- Coenzyme A Synthetase of Yeast. T. Satyanarayana and Harold P. Klein. Bact. Proc., page 158, (1973).

4. Studies on Acetyl- Coenzyme A Synthetase of Yeast: Inhibition by Long-Chain Acyl Coenzyme A Esters. T. Satyanarayana and Harold P. Klein. J. Bact., 115, 600 (1973).

5. Studied on Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein. Proceedings of the IVth International Symposium on Yeasts. Vienna, Austria, A34, (1974).

6. Evidence for Two Immunologically Distinct Acetyl - Coenyzme A Synthetases in Yeast. T. Satyanarayana and Harold P. Klein. Biochem. Biophys. Acta., 341, 396 (1974).

7. Stability Characteristics of "Aerobic" Acetyl- Coenzyme A Synthetase of Yeast. T. Satyanarayana and Harold P. Klein. Fed. Proc., 35, 1668 (1976).

8. Studies on the "Aerobic" Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae: Purification, Crystallation and Physical Properties of the Enzyme. T. Satyanarayana and Harold P. Klein. Arch. Biochem. Biophys., 174, 480 (1976).

9. Comparative Enzymology of Two Distinct Acetyl- Coenzyme A Synthetases of Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein. In IVth International Fermentation Symposium on Yeasts. Montpellier, France, July 1978.

10. Monoclonal Antibody as a Tool for Further Differenciation of the Two Acetyl- Coenzyme A Synthetases (ACS) of Yeast. T. Satyanarayana, Harold P. Klein, Virginia Johnson and Richard A. Goldsby. Fed. Proc., 39, 928 (1980).

11. Subunit Structure and Immunological Distinctness of the Two Acetyl- Coenzyme A Synthetases of Yeast. T. Satyanarayana, Charles H. Chevenka and Harold P. Klein. Biochem. Biophys. Acta., 614, 601 (1980).

12. Naturally Occuring Stabilizing Ligands as a Mode for Further Differentiation of Two Acetyl- Coenzyme A Synthetases (ACS) of Yeast. T. Satyanarayana and Harold P. Klein. Amer. Soc. Microbiol. Meetings, K 20, (1981).

13. Rapid Purification of Fluoroscent Enzymes by Ultra-Filtration. Morris A. Benzaminson and T. Satyanarayana. Prep. Biochem., 1, 24 (1983).

14. The Relationship Between Aerobic Metabolism and the Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein. West Coast Bacterial Physiologists Annual Asilomar Conference, Pacific Grove, California, December 14-16, 1982.

15. Poly-Nucleotide Interactions with the Two Acetyl- Coenzyme A Synthetases of Yeast. T. Satyanarayana and Harold P. Klein (In Preparation).

16. Stability Studies of Isozymes: Part I. Localization of the Stability Factors for the Isozymes of Acetyl- Coenzyme A Synthetases of Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein (In Preparation))

17. Stability Studies of Isozymes: Part II. Identification of the Stability Factor(s) for the Aerobic Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein (In Preparation).

18. Stability Studies of Isozymes: Part III. Characteristics of a naturally occuring Peptide Stabilizing Agent for the Non-Aerobic Acetyl- Coenzyme A Synthetase of Saccharomyces cerevisiae. T. Satyanarayana (In Preparation).

19. Rapid Separation of Interfacing Chromatofocusing Poly Buffer from Peptide Samples of Similar Sizes. T. Satyanarayana, Swedish Marketing Technica, Uppsala, Sweden (1983).

20. LAB NOTES: Hydroxylapatite Facilities Rapid Removal of Polybuffers from Peptide of Closely Related Molecular Weights. T. Satyanarayana, Biologics., Calbiochem-Behring Newsletter 10, 2 (1984).

21. Purification and Properties of Non-Aerobic Acetyl- CoA Synthetase of Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein (In Preparation).

22. Structure and Function of Isozymes in Saccharomyces cerevisiae. T. Satyanarayana and Harold P. Klein. VIth International Symposium on Yeast, July 9-13, Montpellier, France, page VI-3-0 (1984).