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DIHYDROXYACID DEHYDRATASE: THE SITE OF HYPERBARIC OXYGEN POISONING IN BRANCH-CHAIN AMINO ACID BIOSYNTHESIS

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SUMMARY

Exposure of Escherichia coli to hyperbaric oxygen results in rapid inactivation of dihydroxyacid dehydratase but not of other enzymes required for branched-chain amino acid biosynthesis. Unless branched-chain amino acids are supplied, protein synthesis and growth stops abruptly. The sensitivity of dihydroxyacid dehydratase thus accounts for the observed protective role of branched-chain amino acids which cannot be adequately synthesized during exposure to hyperoxia.

INTRODUCTION

Hyperbaric oxygen is toxic for Escherichia coli but certain amino acids, including branched-chain amino acids, provide significant protection. α-Ketoisovaleric acid, an intermediate in the pathway for biosynthesis of branched-chain amino acids, also protects E. coli, and it was proposed that hyperbaric oxygen poisoned one or more of the three enzymes in the parallel reactions for converting pyruvate and α-ketobutyrate, respectively, into α-ketoisovaleric and α-keto β-methylvaleric acids (1). These reactions, as shown for the synthesis of valine, are:

(a) 2 pyruvate $\longrightarrow$ CO_2 + α-acetolactate

(b) α-acetolactate + NADPH $\longrightarrow$ α,β-dihydroxyisovalerate + $NADP^+$

(c) α,β-dihydroxyisovalerate $\longrightarrow$ H_2O + α-ketoisovalerate

α-Ketoisovalerate is converted into valine and into leucine, and α-Keto β-methylvaleric is converted into isoleucine. Poisoning at a single

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enzyme site thus could account for the observed requirement for all three amino acids by E. coli in hyperbaric oxygen (1). We now report that dihydroxyacid dehydratase [EC 4.2.1.9], which catalyzes reaction (c) is the oxygen-sensitive enzyme in the synthesis of branched-chain amino acids.

MATERIALS AND METHODS

E. coli strain K-12, W3389N, obtained from Gholson et al. (2), was grown in synthetic medium (1) with 10 mM quinolinate. Cultures, in exponential growth at 37°C in a stirred vessel, were pressurized with 4 atm of oxygen (gas phase: 1 atm air plus 4 atm oxygen). The vessel was fitted with a separate compartment which contained chloramphenicol. After 10 minutes of exposure to hyperbaric oxygen, the chloramphenicol (final concentration, 150 µg/ml) was tipped into the vessel which was decompressed rapidly and the culture was poured over cracked ice. The cells were removed by centrifugation at 4°C and resuspended in buffer to a concentration of approximately 0.7 grams wet weight of cells in 3 ml. The cells were disrupted by sonication at 4°C for 3 min at maximum power with a Bronwill sonic probe operated for 30 sec intervals with alternate 30 sec intervals for cooling. The disrupted cell suspensions were centrifuged at 4°C for 20 min at 12,000 x g and the supernatants were used for enzyme assays. The extracts were not purified nor dialyzed since the objective was to detect potential oxygen inactivation of specific enzymes and it was desirable to minimize manipulations which might differentially affect enzyme specific activities in control and experimental cultures.

The specific activities of three enzymes were measured. Acetolactate synthase [EC 4.1.3.18], reaction (a); dihydroxyisovalerate dehydrogenase (isomerizing) [EC 1.1.1.89], reaction (b); and dihydroxyacid dehydratase [EC 4.2.1.9], reaction (c) were assayed as described by Desai and Polglase (3) Radhakrishnan, Wagner and Snell (4) and Meyers (5), respectively. Acetoin was measured as described by Westerfeld (6) and keto acids were determined as described by Friedemann and Haugan (7). Protein in the extracts was measured by a modification of the Lowry method as described by Hartree (8). α-acetolactate and α,β-dihydroxyisovaleric acids were synthesized as described by Krampitz (9) and Kiritani and Wagner (10), respectively.

RESULTS AND DISCUSSION

Dihydroxyacid dehydratase was approximately 78% reduced in specific activity in extracts prepared from Escherichia coli cells exposed to 4.2 atm of oxygen for only 10 min (Table 1). Acetolactate synthase specific activity was not significantly altered ($p = 0.01$) under such conditions. There was a small (20%) decrease of doubtful biological significance in dihydroxyisovalerate dehydrogenase (isomerizing) specific activity (Table 1). The amount of α-acetolactate present in each extract prior to the enzyme assay was determined and was subtracted from the data of Table 1. The

TABLE 1 Comparative Effects of Hyperbaric Oxygen Exposure on Three Enzymes of Branched-Chain Amino Acid Biosynthesis in *Escherichia coli*[a]

Enzyme	Specific Activity (U/mg)[b]	
	Air	HPO
Acetolactate synthtase	0.061 ± 0.015	0.074 ± 0.021
Dihydroxyisovalerate dehydrogenase (isomerizing)	10.9 ± 1.36	8.76 ± 1.58[c]
Dihydroxyacid dehydratase	0.836 ± 0.155	0.186 ± 0.035[c]

[a]Cells were exposed to 4.2 atm partial pressure of oxygen (HPO) for 10 min at 37°C during exponential growth and enzyme activities were measured in cell-free extracts as described in the test.

[b]The standard units of activity are: acetolactate synthase, μmoles of acetolactate produced per hr; dihydroxyisovalerate dehydrogenase (isomerizing), μmoles of NADPH oxidized per min; dihydroxyacid dehydratase, μmoles of ketoisovalerate produced per 15 min. Averages ± 1 S.D. are shown for three experiments for each enzyme. The number of separate extracts and total assays, respectively, were: acetolactate synthase (5,26), dihydroxy-isovalerate dehydrogenase (6,16) and dihydroxyacid dehydratase (6,20).

[c]Significantly different at $p \geq 0.01$ from the value for cells with air as the gas phase using Student's t-test.

α-acetolactate concentrations of the extracts from cells grown in air and after 10 min in 4.2 atm of oxygen were not significantly different at P = 0.01 (1.88 ± 0.02 vs 2.61 ± 0.27 nmole/mg protein, respectively). The fact that dihydroxyacid dehydratase was inhibited would not interfere with the assays to measure dihydroxyisovalerate dehydrogenase (isomerizing) since this assay was done spectrophotometrically by measuring the oxidation of NADPH, and the initial slopes over the first 2 min (before end-product inhibition was significant) were used to determine activities.

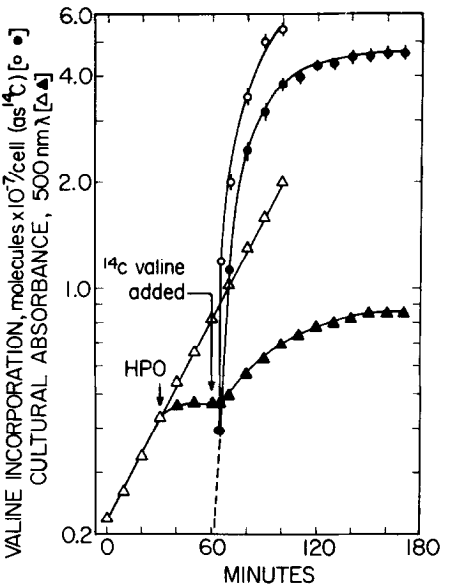

Fig. 1. Incorporation of valine into protein during protection by valine against oxygen poisoning in Escherichia coli. Cultures were grown at 37°C with stirring in a vessel which could be pressurized. The growth medium was minimal salts amino acids (no valine) each at 0.65 mM, and 28 mM glucose as previously described (1). [^{14}C]valine (0.65 x 10^{12} DPM/mole) was added at 60 min to a final concentration of 0.65 mM to cells growing exponentially with air as the gas phase, and (without decompressing the culture) to cells which had been exposed to HPO (a mixture of 1 atm of air plus 4 atm of oxygen) for 30 min to completely stop growth. Samples of the cultures were removed without decompressing the vessel, treated with trichloroacetic acid (5% final concentration), heated, collected on membrane filters, washed with multiple solvents, and counted by liquid scintillation spectrometry as described by Kennell (12). Average + 1 S.D. for 3 determinations for each of 2 experiments are shown for [^{14}C] valine incorporation into protein with air (o) and with HPO (●) as the gas phase. Cultural absorbance was measured with air (△) and with HPO (▲) as the gas phase.

The data of Fig. 1 indicate that the inhibition of the dehydratase enzyme is of biological significance. When cells were exposed to oxygen at 4.2 atm partial pressure while growing in a medium containing 19 amino acids (without valine) there was rapid inhibition of growth (Fig. 1). Upon addition of [^{14}C]valine there was an immediate uptake and incorporation of the valine into trichloroacetic acid-insoluble material accompanied by growth as determined by increase in cultural absorbances (Fig. 1). Thus in hyperoxia, valine provides protection which is accompanied by its uptake and incorporation into protein. After approximately one hr the growth and

incorporation ceased again, suggesting that oxygen-poisoning of other, more resistant, cellular processes had occurred. One may conclude that valine and other branched-chain amino acids protect in hyperoxia because their synthesis is rapidly impaired due to inactivation of dihydroxyacid dehydratase.

A wide variety of life forms are subject to oxygen poisoning by hyperbaric oxygen (11). Because of the rapidity of its loss of activity in hyperoxia and the accompanying cessation of protein synthesis, it appears that inactivation of dihydroxyacid dehyratase is a significant factor in oxygen poisoning in E. coli and perhaps in other life forms when they are dependent on biosynthesis to supply their branched-chain amino acids. This mechanism of toxicity is apparently not important in human cells since they are not known to possess the enzymes to synthesize branched-chain amino acids. The chemical basis for the unusual oxygen sensitivity of dihydroxyacid dehydratase is under study.

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REFERENCES

1. Boehme, D.E., Vincent, K., and Brown, O.R. (1976) Nature 2, 418-420.
2. Gholson, R.K., Tritz, G.J., Matney, T.S., and Andreoli, A.J. (1969) J. Bacteriol. 99, 895-896.
3. Desai, I.D., and Polglase, J. (1965) Biochim. Biophys. Acta, 110, 181-188.
4. Radhakrishnan, A.N., Wagner, R.P., and Snell, E.E. (1960) J. Biol. Chem. 235, 2322-2331.
5. Meyers, J.W. (1961) J. Biol. Chem. 236, 1414-1418.
6. Westerfeld, W.W. (1945) J. Biol. Chem. 161, 495-502.
7. Friedemann, T.E., and Haugan, G.E. (1943) J. Biol. Chem. 147, 415-442.
8. Hartree, E.F. (1972) Biochemistry, 48, 422-427.
9. Krampitz, L.O. (1948) Arch. Biochem. 17, 81-85.

10. Kiritani, K., and Wagner, R.P. (1970) Methods in Enzymology (Tabor, H., and Tabor, C.W., eds.). 17A, pp. 755-764, Academic Press, N.Y. and London.

11. Haugaard, N. (1968) Physiol. Rev. 48, 311-373.

12. Kennell, D. (1967) Methods in Enzymology (Grossman, L., ed.) 12A, pp. 686-693, Academic Press, N.Y. and London.