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AN UNUSUAL TYPE OF REGULATION OF MALATE OXIDASE SYNTHESIS IN *ESCHERICHIA COLI*

A. Hughes Goldie, Suree Narindrasorasak and B. D. Sanwal

Department of Biochemistry, Faculties of Medicine and Dentistry,
University of Western Ontario, London, Canada, N6A 5C1

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SUMMARY:

Mutants of *E. coli* which lack the activity of NAD-specific malate dehydrogenase show the presence of a FAD-dependent malate oxidase which can be assayed using ferricyanide as an electron acceptor. Cells which acquire the ability to form malate dehydrogenase cease to produce detectable levels of malate oxidase. Activity of malate dehydrogenase is definitely required to suppress synthesis of malate oxidase. The substrate and product of malate dehydrogenase, L-malate and oxalacetate, respectively, are apparently not involved in the regulation of malate oxidase. Presence of high derepressed levels of malate oxidase in malate dehydrogenase deficient mutants are determined by a gene linked closely to argG locus. Malate oxidase is produced in small amounts in minimal-glucose medium, but in high concentrations in complex medium. The peak levels are reached in early log phase of growth after which there is a dramatic drop with the approach of stationary phase.

INTRODUCTION:

Apart from the ubiquitous NAD and NADP-dependent malate dehydrogenases, several procaryotes, especially mycobacteria, possess malate oxidases which require FAD as a cofactor (1,2) and use vitamin K (2) as electron acceptor. In several other organisms malate oxidation also occurs through the electron transport chain (3,4) but the actual electron acceptor is not known. In *E. coli* a possible involvement of ubiquinone in malate oxidation has been suggested (3) but malate oxidase as an entity has never been shown to occur in cell free extracts. In experiments aimed at the elucidation of the regulation of gluconeogenesis in *E. coli* we discovered that mutants which lacked NAD-dependent malate dehydrogenase could still grow, albeit slowly, on malate as a sole carbon source. This led to the suspicion that *E. coli* may have a malate oxidase which is able to transfer electrons to the respiratory chain. During the course of investigation of this possibility we found that malate oxidase is induced only in cells which lack completely the activity of the NAD-specific malate dehydrogenase (5). This paper documents the presence and regulation of the malate oxidase (EC 1.1.3.3) in *E. coli*.

MATERIALS AND METHODS:

Genetic Experiments - The genetic strains used are listed in Table 1.

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TABLE 1 Strains of *ESCHERICHIA COLI*

Strain Designation	Genotype	Mutagen
EC 6	Hfr (transposed lac^+), Δ (lac, pro)	
G 6	Hfr, his	
UH-Ac 2	aceE, trp	
Ac 10	aceF	
JRG 590	HfrH, Δ (nad C - aceF)	
JRG 596	Hfr H Δ (aroP - aceF)	
HG 29	Hfr H, leu, pps, argG, strA	
HG 33	Derivative of HG 29, mdh	NNG
HG 39	Derivative of HG 29, mdh	NNG
HG 37	Derivative of EC 6, mdh	mu-1
HG 38	Derivative of EC 6, mdh	mu-1

NNG is N-methyl-N'-nitro-N-nitrosoguanidine; mu-1 is phage mu-1. The genotypes described above are only those relevant to the present work. Symbols represent: lac, no growth on lactose; his, trp, arg, leu, nad growth requirement for histidine, tryptophane, arginine, leucine and nicotininc acid; ace E and ace F, absence of pyruvate dehydrogenase and dihydrolipoamide transacetylase component, respectively, of the pyruvate dehydrogenase complex.

The strains with the prefix HG were made in the laboratory. Rest of the strains were obtained from the E. coli Genetic Stock Center, New Haven. Cells were grown as needed in Luria Broth (LB medium) or in minimal medium (6) supplemented with 0.4% carbon source. Mutants lacking malate dehydrogenase (EC 1.1.1.37) were recognized initially by their slow growth on media containing L-malate as the sole carbon source, as described by Courtright and Henning (7). Mutagenesis was performed by the use of mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (8) or the use of bacteriophage mu-1 (9).

Enzyme Assays - Cell free extracts were prepared either by sonication or by passing the cells through a French Press. For all assays except pyruvate oxidase, the cells were washed and suspended in 0.05M Tris-HCl, pH 7.5. For the assay of pyruvate oxidase the cells were suspended in 0.15M phosphate buffer, pH 6.5 before sonication. Malate dehydrogenase was measured in cell extracts by a previously published procedure (5). Pyruvate oxidase was assayed spectrophotometrically by using ferricyanide as an artifical electron acceptor (10). Fumarase and succinate dehydrogenase were estimated using standard procedures. Malate oxidase was assayed in mixture containing 5 μmole sodium malate, 1 μmole potassium ferricyanide, 10 μmole KCN, 100 μmole KCl and 100 μmole Tris-HCl, pH 7.5 in a total volume of 1.0 ml. The reduction of ferricyanide was monitored at 400 nm. Specific activities of all enzymes are defined as μmoles of product formed per min per mg protein.

RESULTS AND DISCUSSION:

Malate oxidase is present, if at all, in negligible amounts in cells which have a functional malate dehydrogenase (Table 2). Mutants which lack the activity of malate dehydrogenase completely, produce considerable amount of malate oxidase. This is seen from an inspection of Table 2. Three

TABLE 2 Malate Oxidase and Malate Dehydrogenase Activity in Various Strains

Strain	Malate Oxidase		Malate Dehydrogenase	
	LB medium[a]	minimum[b] medium	LB medium	minimum medium
EC 6	-	0	10.9	10.2
HG 37	0.16	0.07	0	0
HG 38	-	0.03	0	0
HG 29	0	0	9.6	12.0
HG 33	1.43	0.15	0	0
HG 34	-	0	-	1.2

[a] Cells were grown in LB medium supplemented with 0.1% glucose and harvested at the mid-log phase.

[b] Cells grown in minimal medium containing 0.4% glucose were harvested at the end of log phase.

independently isolated mutants using two different procedures, viz., chemical and phage mutagenesis, and totally deficient in malate dehydrogenase activity produce high, but variable amounts of malate oxidase. A fourth mutant (HG 39) which probably has an altered malate dehydrogenase and has only low levels of this enzyme does not produce malate oxidase. A complete absence of malate dehydrogenase activity thus seems obligatory for the induction of malate oxidase. This finding is a common one with other malate dehydrogenase mutants which we have cursorily examined but which are not listed here. To probe further into the question of how general this linkage between the absence of one enzyme and induction of another may be, we tried to obtain revertants of the various mutants at the mdh locus by plating cells in malate-minimal medium and looking for fast growing colonies. For unknown reasons, however, invariably all such fast-growing colonies appearing on malate medium were still deficient in malate dehydrogenase. Fast growth of such colonies may be due to derepression of several other malate utilizing enzymes, such as the NAD (EC 1.1.1.38) and NADP-specific (EC 1.1.1.40) malic enzymes known to be present in *E. coli* (5). We have, however, not pursued this point further. In the absence of a reliable selection method to obtain mdh^+ revertants, we introduced the mdh^+ marker into mdh mutants by mating procedures. Since it is known that mdh is linked to argG (11) we mated phenocopies of strain HG 33 carrying the markers mdh and argC (Table 1) with Hfr G6 and selected for $argG^+$ recombinants. Almost 60% of these were mdh^+ in accord with published data (11). In all of the eight $argG^+$, mdh^+ recombinants tested, only negligible amounts of malate oxidase were present, while in other seven $argG^+$, mdh recom-

binants the specific activity of malate oxidase tested in mid-log phase in LB medium was around 0.12. It, therefore, does seem to be a fair generalization that malate oxidase appears only in cells which lack malate dehydrogenase activity. The only inexplicable result with the argG$^+$, mdh recombinants was the presence of low level of malate oxidase in these cells (specific activity about 0.12) compared to the levels of this enzyme in the parent (specific activity 1.43, Table 2). Indeed, in most independently generated mdh mutants, the specific activity of malate oxidase in LB medium is found to be between 0.10 to 0.18. HG 33 (Table 2) is an exception in possessing ten times higher activity. Since this mutant was made by the use of chemical mutagen, it seemed possible that it carried also mutation of a regulatory gene involved in the synthesis of malate oxidase and linked very closely to the argG locus. To test this hypothesis we transduced strain HG 33 with P_1 phage lysate grown on Hfr H (wild-type) and selected transductants which were arg$^+$. In all three of the mdh, argG$^+$ transductants studied the levels of malate oxidase at log phase were in the range of 0.12 - 0.18 just like the sexual recombinants discussed before. It thus seems probable that along with its regulation by mdh, malate oxidase is also regulated by the product of a gene closely linked to arg G.

In addition to regulation by gene products some unknown metabolites seem also to participate in the synthesis of malate oxidase. This is evident from Table 2. The specific activity of the enzyme in cells grown on minimal medium containing glucose is much lower than in those grown in complex media. Since malate dehydrogenase is involved in the regulation of malate oxidase, it seems possible that the substrate or product of this enzyme may be the actual modulator of malate oxidase. In all of the malate oxidase producing strains tested (i.e. mdh strains) malate and L-aspartate (which would indirectly generate oxalacetate by transamination) tested singly or included in the minimal or LB media at concentrations of up to 10 mM during growth, however, caused no significant change in the specific activity of the enzyme compared to the controls. It would thus appear that malate dehydrogenase is directly involved in regulation in as yet unknown way.

In order to generate further clues regarding the mechanism of malate oxidase regulation, we enquired what the function of this enzyme might be. One distinct possibility was that malate oxidase is linked with other reactions of the tricarboxylic acid cycle and functions essentially as malate dehydrogenase. The second possibility was that it may be concerned with the uptake and transport of diverse substrates from the medium into the interior of the cell, like some other membrane-bound oxidases (12). To investigate the first possibility further we followed the synthesis of malate oxidase and several

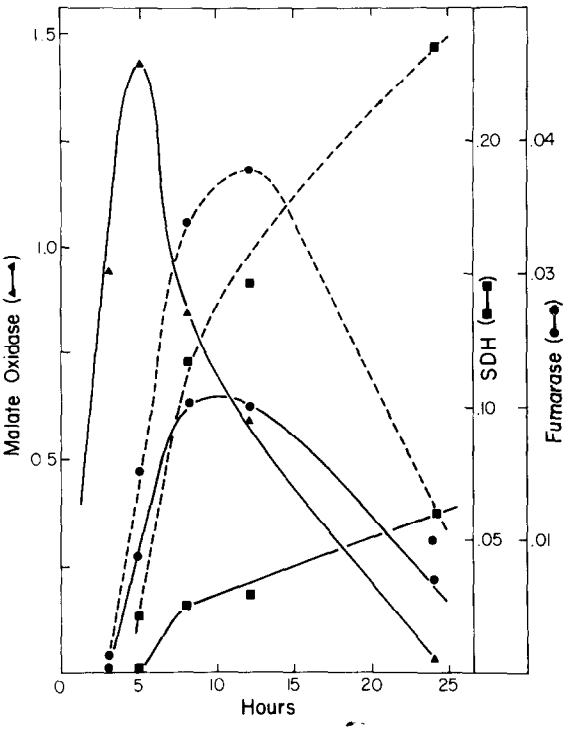

Figure 1. The activity of malate oxidase, succinate dehydrogenase and fumarase as a function of the time of growth of cells. Solid lines represent HG 33 and broken lines HG 29. The cells were grown in LB medium supplemented with 0.1% glucose.

crucial enzymes of the tricarboxylic acid cycle during growth of various strains on LB medium. These results are presented in Figure 1. It will be noted that malate oxidase is induced in the mdh mutant HG 33 in the early log phase of growth and reaches a peak at the late log phase after which its activity precipitously declines with the onset of the stationary phase. Other enzymes of the tricarboxylic acid cycle, malate dehydrogenase (not shown), fumarase and succinate dehydrogenase begin to increase only at mid-log and reach a peak well after the beginning of the stationary phase. For inexplicable reasons, the specific activities of fumarase and succinate dehydrogenase remain quite low in mutants which possess malate oxidase (Figure 1). These results cannot be considered as conclusive evidence but do make the possibility less likely that malate oxidase functions as part of the tricarboxylic acid cycle. It also, directly or indirectly, does not seem to participate in gluconeogenesis, an event which should be of some significance when cells are growing in a complex medium. We (Goldie and Sanwal, unpublished) have evidence that the crucial enzyme of gluconeogenesis, phosphoenolpyruvate

carboxykinase, is induced only when the cells reach the stationary phase, at a time when hardly any malate oxidase is synthesized in the cells. We favour the hypothesis at the moment that malate oxidase may be coupled to the electron transport chain and may be instrumental in the generation of proton gradients for solute translocation. Studies to test this hypothesis are in progress.

The regulation of malate oxidase described here is strikingly reminiscent of another similar enzyme in *E. coli*, namely, pyruvate oxidase (13). It has been reported that the activity of pyruvate oxidase in a mutant lacking pyruvate dehydrogenase complex is derepressed (13). In view of our findings with malate oxidase we considered it desirable to investigate whether various other mutants of the complex behave in a similar manner, i.e., have derepressed levels of pyruvate oxidase. Strains lacking either pyruvate dehydrogenase (UH-Ac 2, Table 1) or dihydrolipoamide transacetylase (Ac 10) or both (JRG 590) had levels of pyruvate oxidase comparable to the wild-type (EC 6 and G 6). It is thus clear that pyruvate oxidase despite its functional similarity to malate oxidase is not regulated in the same manner as malate oxidase itself.

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REFERENCES:

1. Tobari, J. (1964) Biochem. Biophys. Res. Commun. 15, 50-54.
2. Iwai, K., and Brodie, A.F. (1973) J. Biol. Chem. 248, 7487-7494.
3. Cox, G.B., Snoswell, A.M., and Gibson, F. (1968) Biochim. Biophys. Acta, 153, 1-12.
4. Jurtshuk, P., Bednarz, A.J., Zey, P., and Denton, C.H. (1969) J. Bacteriol. 98, 1120-1127.
5. Sanwal, B.D. (1969) J. Biol. Chem. 244, 1831-1837.
6. Miller, J.H. (1972) Experiments in Molecular Genetics, pp. 431-435, Cold Spring Harbor Laboratory, New York.
7. Courtright, J.B., and Henning, U. (1970) J. Bacteriol. 102, 722-728.
8. Adelberg, E.A., Mandel, M., and Chen, G.C.C. (1965) Biochem. Biophys. Res. Commun. 18, 788-795.
9. Bukhari, A.I., and Metlay, M. (1973) Virology 54, 109-116.
10. Russell, P., Hager, L.P., and Gennis, R.B. (1977) J. Biol. Chem. 252, 7877-7882.
11. Heard, J.T., Jr., Butler, M.A., Baptist, J.N., and Matney, T.S. (1975) J. Bacteriol. 122, 329-331.
12. Kaback, H.R. (1974) Science, 186, 882-892.
13. Gennis, R.B., and Hager, L.P. (1961) J. Biol. Chem. 236, 1013-1018.