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ESSENTIAL CARBOXYL RESIDUES IN YEAST ENOLASE[1]

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Yeast enolase (EC 4.2.1.11) is rapidly inactivated at pH 6.1 by three different water-soluble carbodiimides -- 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate, and 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide iodide. Inactivation is most likely due to the modification of essential carboxyl residues at the enzyme active site.

Chemical modification studies have shown that several amino acid residues are essential for the catalytic activity of yeast enolase. The enzyme has a single essential arginyl residue per subunit (1-3) which is likely involved in binding the anionic phosphate moiety of substrates to the active site (2). In addition, enolase has been shown to contain essential histidyl residues (4-6), but the role played by histidine is less well defined. Although the inactivation of enolase has been correlated with the modification of methionyl residues (7,8) and cysteinyl residues under denaturing conditions (9), in neither instance are the modified residues thought to be involved in the catalytic mechanism. Similarly, lysyl residues are not likely involved in substrate binding or catalysis (6).

It has been shown that glycidol phosphate, a substrate analogue, inactivates rabbit muscle enolase by reaction with a carboxyl group at the active site (10). However, this reagent fails to inactivate yeast enolase (11). We have modified yeast enolase with several different carbodiimides, reagents known to be highly selective for the modification of carboxyl residues in proteins (12-15). Our study suggests that yeast enolase indeed has essential carboxyl groups.

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[2]Taken in part from the senior Independent Study thesis of A. L. G., The College of Wooster, 1978.

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MATERIALS AND METHODS

2-Phosphoglycerate (2-PGA)[4], 3-PGA, EDC, CMC, and MES were obtained from Sigma. Methyl iodide was purchased from Matheson, Coleman, and Bell, and G-6-P was from Boehringer Mannheim. Yeast enolase, from Sigma, was characterized and assayed as previously described (2). EAC was synthesized from EDC and methyl iodide by published procedures (16). Chemical modifications of enolase were carried out at 25° under the conditions given in the figure and table legends. Modifications were usually initiated by the addition of a freshly prepared stock solution of carbodiimide in buffer to a solution of enzyme, in some cases together with substrates, inhibitors, or G-6-P, in the same buffer. In some experiments, carbodiimides were pre-incubated in buffer with substrates, inhibitors, or G-6-P for various times before initiating the modification by addition of enzyme in order to determine the effect of phosphorylated compounds on the carbodiimide. Aliquots were periodically withdrawn, assayed, and compared to a control subjected to the same conditions but in the absence of carbodiimide. In some cases, aliquots were passed through a column (0.9 x 40 cm) of Bio-Gel P-6 to remove excess reagents, assayed for specific activity, and hydrolyzed for 18 hr in 6 N HCl at 105° and subjected to amino acid analysis on a Beckman 120C amino acid analyzer.

RESULTS

Yeast enolase is rapidly inactivated by CMC, EDC, and EAC, with the rate of inactivation markedly dependent on the carbodiimide employed (Figure 1). When enolase is modified by 20 mM carbodiimide in 50 mM MES, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 6.1, EAC is the most effective inactivating agent, followed by EDC and CMC, in that order. Under these conditions, 70% inactivation is achieved after 9 min with EAC, 29 min with EDC, and 56 min with CMC.

Although carbodiimides are highly selective for the modification of carboxyl residues in proteins at slightly acidic pH (12,13), cysteine and tyrosine can also be modified to some extent under these conditions (13,17). Enolase has only one cysteine per subunit which is unreactive towards modifying reagents except under denaturing conditions (9), thus it is very unlikely that inactivation by carbodiimides is due to the modification of this cysteine. This is supported by the fact that amino acid analysis of enolase which was 95% inactivated by EDC shows no loss of cysteine. Tyrosine can also react with carbodiimides to form O-arylisoureas which are very stable to acid hydrolysis (17). Amino acid analysis of enolase which was 65% inactivated by EDC at pH 6.1 shows no loss of tyrosine relative to the control,

[4] Abbreviations used are: PGA, phosphoglycerate; MES, 2-(N-morpholino)-ethanesulfonic acid; G-6-P, glucose-6-phosphate; P_i, inorganic phosphate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate; EAC, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide iodide (also known by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide methiodide, a name less descriptive of the actual structure of the compound).

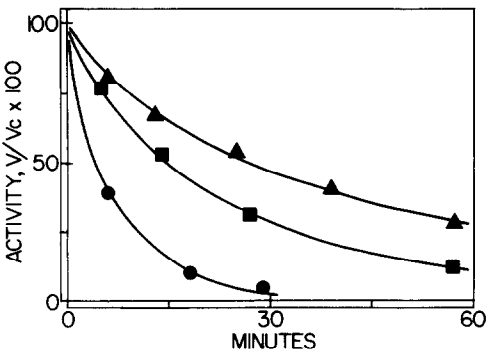

FIGURE 1: Yeast enolase inactivation by carbodiimides. Enolase, 1 μM, was modified by 20 mM carbodiimide in 50 mM MES, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 6.1. The carbodiimides used were (●) EAC, (■) EDC, and (▲) CMC. The control retains full activity for the duration of the experiment.

while a sample which was 95% inactivated shows only 0.2 less tyr per subunit (data not shown). These data eliminate the possibility that inactivation by EDC is due to the modification of cysteinyl or tyrosyl residues, and strongly suggest that carbodiimides inactivate enolase by modifying carboxyl residues.

The reaction of carbodiimides with carboxyl groups is thought to form an intermediate O-acylisourea which can then either rearrange to a more stable N-acylurea, undergo transfer to a suitable amine nucleophile, or hydrolyze to regenerate the carboxyl group (12,13). The formation of a stable N-acylurea is indicated by our observation that if enolase is 95% inactivated by EDC, then excess reagent is removed by gel filtration, no reactivation occurs within 3 hr. Further evidence for the formation of an N-acylurea is our observation that neither glycinamide hydrochloride nor semicarbazide hydrochloride, at 0.4 M, cause any acceleration of the rate of inactivation of enolase by 20 mM EDC at pH 6.1.

All substrates and, with the exception of P_i (2), competitive inhibitors of enolase contain both phosphate and carboxylate functionalities (8). Since carbodiimides react with phosphate and carboxyl moieties of organic compounds (18,19), protection experiments with enolase must be interpreted with care. If EAC is pre-incubated for 45 min at pH 6.1 with either 3-PGA or P_i, competitive inhibitors of enolase, little or no inactivation is observed after 1 hr of modification (Table I). In the absence of competitive inhibitors, enolase is 99% inactivated under these conditions. However, that protection may only be apparent and is

TABLE I: Effect of Phosphorylated Compounds on the Inactivation of Yeast Enolase by EAC. Effect of Pre-incubation[a]

Added Compound	% Inhibition
None	99
P_i	0
3-PGA	5
G-6-P	15

[a]EAC, 25 mM, was pre incubated for 45 min with either 125 mM P_i, 25 mM 3-PGA, or 25 mM G-6-P in 50 mM MES, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 6.1. Modification was then initiated by addition of an aliquot of enzyme in 0.25 volume of buffer, giving final concentrations of 1 μM enolase, 20 mM EAC, 20 mM 3-PGA or G-6-P, and 100 mM P_i. After 1 hr an aliquot was assayed for enzyme activity relative to a control subjected to the same conditions but in the absence of EAC.

TABLE II: Effect of 3-PGA on the Inactivation of Yeast Enolase by EAC. Effect of Time of Pre-incubation of EAC with 3-PGA[a]

Time of Pre-incubation, min	% Activity
0	32
10	58
20	82
30	100

[a]Conditions the same as in Table I, except that EAC was pre-incubated with 3-PGA for the indicated time before initiating modification by adding enzyme. Enzyme activity was determined after 30 min modification.

likely due to the reaction of the phosphate and/or carboxyl moities of these compounds with carbodiimide, thus reducing the effective concentration of modifying agent, is suggested by the fact that G-6-P, which is not an inhibitor of enolase, also provides almost complete protection against inactivation (Table I).

Table II suggests that the reaction of carbodiimides with 3-PGA is time dependent. If enolase is modified by 20 mM EAC in the presence of 20 mM 3-PGA, the extent of inactivation is dependent on the order of addition of reagents and the time of pre-incubation of EAC with 3-PGA. If EAC and 3-PGA are pre-incubated for 0, 10, 20, and 30 min before the addition of enzyme, activity after 30 min

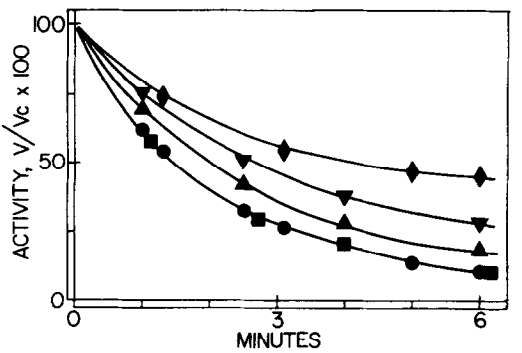

FIGURE 2: Protection against EAC inactivation of yeast enolase. Enolase, 1 μM, in 50 mM MES, 1 mM $MgCl_2$, 0.01 mM EDTA, pH 6.1, was modified by 40 mM EAC, either in the absence (●) of added phosphorylated compounds, or in the presence of (■) 40 mM G-6-P plus 40 mM acetate, or (▲) 40 mM P_i, or (▼) 40 mM 3-PGA, or (◆) 40 mM 2-PGA. In each instance, the modification was initiated by adding an aliquot of a freshly-prepared stock solution of EAC to the enzyme plus phosphorylated compound.

modification is 32%, 58%, 82%, and 100% of the control, respectively. This suggests that protection experiments may be possible if modification times are brief and concentrations of carbodiimide and substrate (inhibitor) are comparable. The results of such an experiment are shown in Figure 2. If enolase is inactivated by by 40 mM EAC at pH 6.1, activity is reduced to 10% after 6 min. G-6-P plus acetate, 40 mM each, provide no protection against inactivation (compare to Table I). P_i provides a small amount of protection, 3-PGA affords more protection, and substrates provide the greatest degree of protection. While exact interpretation of this data is difficult, it suggests that inactivation of yeast enolase by carbodiimides is likely due to the modification of carboxyl residues at the enzyme active site.

DISCUSSION

The data suggest that yeast enolase has essential carboxyl residues. Since 2-PGA affords the greatest degree of protection against inactivation by carbodiimides (Figure 2), it is likely that inactivation is due to the modification of carboxyl groups at the active site of the enzyme. This conclusion is made somewhat tenative by our observation that G-6-P, which is neither a substrate nor competitive inhibitor, affords significant protection under the appropriate conditions (Table I). However, this protection by G-6-P is likely due to the reaction of its phosphate moiety with the carbodiimide, thus reducing the effective concentration of modifying

agent. A survey of the literature suggests that little attention is given to this undesirable side reaction of phosphate moieties with carbodiimides in protein modification studies; in fact, several studies use phosphate buffer for modification studies (20, 21). A number of studies suggest that low concentrations of phosphorylated substrates fail to protect against inactivation by high concentrations of carbodiimides (20, 22). Such results are usually interpreted that inactivation does not involve modification of carboxyl residues at the binding site of these substrates, when in actuallity the effective concentration of substrate may be rapidly reduced by carbodiimide to a level where no protection is possible. Carbodiimides are well known to react with phosphate functionalities and activate them towards nucleophilic attack (23). Thus, in cases where phosphorylated substrates are reported to accelerate the inactivation of enzymes by carbodiimides (24), it is possible that the carbodiimide-activated substrate undergoes attack by a nucleophilic group on the enzyme, the substrate becoming covalently attached with concomitant inactivation. Our work suggests that attention must be given to conditions when one attempts to use substrates and inhibitors containing phosphate and/or carboxyl moieties to protect against inactivation of enzymes by carbodiimides.

Finally, either EDC or CMC has been used in almost every report of the inactivation of an enzyme by a carbodiimide. The use of EAC has been reported only once, and that in preliminary form (25). We find it very interesting that EAC inactivates yeast enolase much more rapidly than either EDC or CMC. This increased reactivity, coupled with the ease with which the ^{14}C-labelled reagent may be synthesized in one step (16) from commercially available EDC, suggests that EAC may prove more useful for protein modification studies than either EDC or CMC. We are currently further characterizing the inactivation of enolase by EAC.

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REFERENCES

1. Riordan, J.F., McElvany, K.D. and Borders, C.L., Jr. (1977) Science, 195, 884-886.

2. Borders, C. L., Jr., Woodall, M. L. and George, A. L., Jr. (1978) Biochem. Biophys. Res. Comm., 82, 901-906.
3. Elliott, J. I. and Brewer, J. M. (1978) Arch. Biochem. Biophys., 190, 351-357.
4. Westhead, E. W. (1965) Biochemistry, 4, 2139-2144.
5. Elliott, J. I. and Brewer, J. M. (1978) Fed. Proc., 37, 1813.
6. George, A. L., Jr. and Borders, C. L., Jr. (1979) submitted for publication.
7. Brake, J. M. and Wold, F. (1962) Biochemistry, 1, 386-391.
8. Wold, F. (1971) The Enzymes, 3rd Ed., 5, 499-538.
9. Oh, S.-K., Travis, J. and Brewer, J. M. (1973) Biochim. Biophys. Acta, 310, 421-429.
10. Schray, K. J., O'Connell, E. L. and Rose, I. A. (1973) J. Biol. Chem., 248, 2214-2218.
11. Shen, T. Y. S. and Westhead, E. W. (1973) Biochemistry, 12, 3333-3337.
12. Hoare, D. G. and Koshland, D. E., Jr. (1967) J. Biol. Chem., 242, 2447-2453.
13. Carraway, K. L. and Koshland, D. E., Jr. (1972) Methods Enzymol., 25, 616-623.
14. Lin, T.-Y. and Koshland, D. E., Jr. (1969) J. Biol. Chem., 244, 505-508.
15. Riordan, J. F. and Hayashida, H. (1970) Biochem. Biophys. Res. Comm., 41, 122-127.
16. Sheehan, J. C. Cruickshank, P. A. and Boshart, G. L. (1961) J. Org. Chem., 26, 2525-2528.
17. Carraway, K. L. and Koshland, D. E., Jr. (1968) Biochim. Biophys. Acta, 160, 272-274.
18. Khorana, H. G. (1953), Chem. Rev., 53, 145-166.
19. Kurzer, F. and Douraghi-Zadeh, K. (1967) Chem. Rev., 67, 107-152.
20. Huc, C., Olomucki, A. and Thomé-Beau, F. (1975) F. E. B. S. Lett., 60, 414-418.
21. Pho, D. B., Roustan, C., Tot, A. N. T. and Pradel, L.-A. (1977) Biochemistry, 16, 4533-4537.
22. Swaisgood, H. and Natake, M. (1973) J. Biochem. (Tokyo), 74, 77-86.
23. Khorana, H. G. (1961) Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest, John Wiley and Sons, New York.
24. Ariki, M. Fukui, T. (1978) J. Biochem. (Tokyo), 83, 183-190.
25. Timkovich, R. (1977) Anal. Biochem., 79, 135-143.