DUAL CONSEQUENCE OF MUTATION IN THE DIHYDROFOLATE REDUCTASE GENE OF DIPLOCOCCUS PNEUMONIAE

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Received June 26, 1969

Ten mutations have been identified at four genetically linked sites on the D. pneumoniae chromosome which determine an increase in the amount of dihydrofolate reductase and severely alter the structure as well. Mutant enzyme levels are increased as much as 60-fold over the wild-type. The mutant enzymes are markedly altered in the ability to bind the antifolate, amethopterin, and in respect to their thermostability properties. These findings may be pertinent to the question of the role of the structural gene itself in setting limits of synthesis of the gene product.

The occurence of mutation in the dihydrofolate reductase structural gene of Diplococcus pneumoniae which affects the structure and at the same time determines an increase in the amount of the corresponding enzyme, was reported from this laboratory some time ago (Sirotnak et al., 1964a; 1964b; 1964c). Evidence that this dual-effect was a general property of many of the mutations occurring at numerous sites within the gene was presented more recently (Sirotnak and Hachtel, 1969; Sirotnak et al., 1969). The idea that a structural gene mutation can alter upward the level of the enzyme for which it is encoded seemed important in that it further emphasizes a possible role of the gene itself in fixing limits of synthesis. The biochemical evidence for individual mutational effects on the structure of dihydrofolate reductase, with two exceptions, was somewhat tenuous, consisting of a subtle decrease in the bind-

ing by enzyme of the antifolate, amethopterin (Sirotnak et al., 1969). More conclusive evidence that the quantitative effects on enzyme concentration were related to widespread alteration of the structural gene has now been obtained. Ten mutations have recently been identified at four different sites within the dihydrofolate reductase structural gene. These mutations all appear to determine a 60- fold increase in the amount of dihydrofolate reductase and at the same time markedly alter, not only the binding of antifolate, but the stability of the enzyme as well. Details of the genetic analyses and the biochemical findings for these extreme dual-effect mutants will be presented in a forthcoming communication (Sirotnak, Williams and Vitaglione, manuscript in preparation).

Individual mutant strains, each bearing a single mutation, were isolated on the basis of resistance to amethopterin. Methods of isolation, identification and genetic analysis have already been given (Sirotnak et al., 1964d; Sirotnak and Hachtel, 1969). The various biochemical procedures have also been described in detail (Sirotnak et al., 1964b). Determinations for dihydrofolate reductase activity in crude extract were similar to that of Osborn and Huennekens (1958). Enzyme concentration and amethopterin binding experiments were carried out by direct titration of enzyme activity in crude extract in a manner (Sirotnak et al., 1964b) similar to that of Ackermann and Potter (1949) or Werkheiser (1961).

The biochemical properties associated with the 10 dual-effect mutations are compared to the wild-type enzyme activities in Table 1. All of these mutations determine a high level of enzyme activity. This was found to be 60- fold in at least two cases. A similar level of enzyme activity is assumed to be determined by the other mutations, but is apparently not demonstrable

because of the relatively high lability of these enzymes. As in the case of the two mutations, the assumed specific activities determined by the others are in agreement with the relative resistance levels of 60-fold exhibited by all of the corresponding mutant strains. In any event, the overall concordance seen between relative enzyme activity and relative enzyme concentration, as determined by drug titration, renders unlikely the possibility that increased enzyme activity in some cases may be due solely to an increase in turnover rate.

From an examination of the data on drug inhibition or heat inactivation of enzyme activity (Table 1), it is clear that the mutant enzymes are different from the wild-type enzyme. Because of the high affinity for amethopterin, inhibition of the wild-type enzyme is stoichiometric, but only until the concentration of drug approaches a molar equivalent concentration of enzyme and drug (Sirotnak et al., 1964b), where the residual activity is about 9 percent of the control without drug. At this concentration the mutant enzyme activity remaining is 2- to 3- times greater, indicating a marked decrease in the binding of the drug. The relative instability of the mutant enzymes, when compared to the wild-type, was demonstrated by heating each crude extract at 55 C for 5 minutes. A more pronounced effect was obtained with all of the mutant enzymes. In most cases, the inactivation was quite severe (as much as 20-fold more mutant enzyme inactivation than wild-type). That the differences found in the thermostability of wild-type and mutant enzymes were actually due to structural dissimilarities, rather than to other differences between the extracts, was shown by conventional mixing experiments. Inactivation of mixtures of mutant and wild-type extract was always equal to the sum of the inactivation obtained individually.

Table 1. The properties and relative amount of dihydrofolate reductase from mutant and wild-type D. pneumoniae.

Strain	Relative specific activity[a]	Relative enzyme conc.[b]	Drug inhibition[c] (Residual activity) %	Heat inactivation[d] (Residual activity) %
wild-type	1.0	1.0	9	84
ame^r- 71	40.0	35.0	24	26
ame^r- 74	11.2	9.4	25	4
ame^r- 75	29.0	32.0	17	73
ame^r- 77	9.2	10.4	25	5
ame^r- 78	69.0	73.0	20	17
ame^r- 88	37.0	40.6	22	59
ame^r- 89	58.0	60.5	30	70
ame^r- 94	9.9	11.4	21	12
ame^r- 97	6.9	6.8	26	63
ame^r- 98	14.0	15.7	25	40

[a] Relative rate of TPNH dependent reduction of folate-H_2 per mg protein.

[b] Relative amount of amethopterin bound per mg protein.

[c] Residual enzyme activity at molar equivalent concentration of enzyme and drug.

[d] Residual activity remaining after heating enzyme preparations (1 mg of protein per ml diluted in 0.05M potasium phosphate buffer, pH 7.4) for 5 min. at 55 C.

This form of dual biochemical effect of mutation is similar to that observed earlier in the L-arabinose operon of Escherichia coli (Lee and Englesberg, 1963). It remains unclear as to how a "missense" alteration of a structural gene could increase the cellular level of the corresponding

enzyme. A likely possibility is that such mutations in some way set higher limits of synthesis (Ames and Martin, 1964; Stent, 1964). In view of the possible relevance of these findings to protein synthesis in general, we are continuing these studies in an effort to determine the level(s) of information transfer involved.

ACKNOWLEDGEMENT

The author gratefully acknowledges the interest, encouragement and helpful advice of Doctor Dorris J. Hutchison during the course of these studies. This work was supported in part by grant CA-08748 from the National Cancer Institute and grant T-107 from the American Cancer Society.

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