GEL FILTRATION PROPERTIES OF MUTANT AND WILD-TYPE DIHYDROFOLATE REDUCTASE FROM DIPLOCOCCUS PNEUMONIAE*

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Received May 3, 1965

The isolation of amethopterin-resistant strains of Diplococcus pneumoniae has permitted the selection for mutants showing an alteration in the structure or synthesis of dihydrofolate reductase (Sirotnak, Lunt and Hutchison, 1964; Sirotnak, Donati and Hutchison, 1964a, 1964b). One mutant, among a variety examined, is of more than usual interest. This mutant is approximately 100-times more resistant than the wild-type and bears a single mutation (ame^r-3), apparently within the structural cistron for dihydrofolate reductase. The mutation determines a more heat labile form of the enzyme and although it has not been found to alter catalytic properties, it occurs at a site on the DNA almost adjacent to the site of another mutation which does. Of additional significance is the fact that the ame^r-3 mutation also determines a 120-fold increase in dihydrofolate reductase synthesis. We have examined this enzyme in both ame^r-3 mutant and wild-type preparations by gel filtration. The results, which are presented here, reveal a striking difference in elution properties of the dihydrofolate reductase in these strains.

Methods of genetic analysis, cultural conditions for the various strains and the preparation of enzymatically active cell-free extracts

*This work was supported in part by grant CA 03192-07 from the National Cancer Institute, National Institutes of Health, Bethesda, Maryland, and by grant T-107 from the American Cancer Society.

‡U.S.P.H.S. Research Career Development Awardee (1-K3-CA-28,199-01).

have been described (Sirotnak, Lunt and Hutchison, 1964; Sirotnak, Donati and Hutchison, 1964). Folate-H_2 was prepared by the Blakley (1960) modification of the method of Futterman (1957). Enzyme activity was determined in a manner similar to that of Osborn and Huennekens (1958) by measuring the decrease in absorbancy (340 mμ) at 25°C when 0.033 mM folate-H_2 and 0.065 mM TPNH were combined with enzyme extract in 0.05 M potassium phosphate buffer (pH 7.3) containing 12.8 mM mercaptoethanol.

Sephadex G-100 and G-200 (Pharmacia) or Biogel P-300 (Bio-Rad) were equilibrated in 0.05 M Tris buffer (pH 7.4) containing 1 mM EDTA. The gel was then packed in buffer in a glass cylinder (I.D. ≈ 3 cm), equipped at the bottom with a coarse sintered glass filter, until a bed height of 47 cm was attained. Before adding the sample, the column was further equilibrated under hydrostatic pressure by attaching a reservoir of buffer and washing for 24-48 hours. When adding the sample (1 to 10 ml of enzyme extract in buffer) the liquid level was brought to the level of the gel surface. Sample was washed into the gel with 3 ml of buffer, additional buffer then added and the reservoir attached, restoring hydrostatic pressure. Fractions of 7.4 ml were collected, assayed for enzyme activity and the protein content of each determined spectrophotometrically at 280 mμ.

Analytical utilization of the gel column requires a determination of the void volume, i.e., the liquid volume external to the gel particles (Flodin, 1962). This was obtained by following the elution of large marker proteins that are excluded from the gel. This value was also coincident with the eluate volume in which the majority of the bulk protein from the crude extract comes off the column (exclusion front) and was characterized by a sharp elution peak. Wild-type enzyme in crude preparations on G-100 Sephadex (Fig. 1A) was eluted at a volume (V) twice the void volume (V_o). A linear relationship

exists between the logarithm of molecular weight of a protein and the ratio V/V_0 (Whitaker, 1963). By graphically comparing the ratio for this enzyme to values for proteins of known molecular weight, the wild-type enzyme was found to have a molecular weight of approximately 20,000. This is similar to values derived for other bacterial (Albrecht, A. M. private communication) and animal (Zakrzewski, 1965; Kaufman and Gardiner, 1965) dihydrofolate reductases.

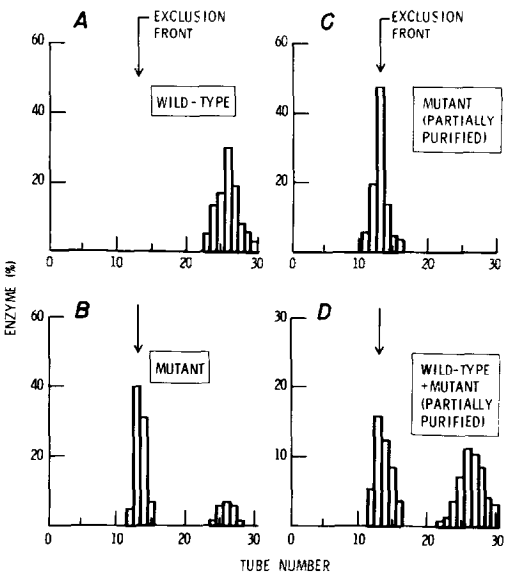

Fig. 1. Elution properties of mutant and wild-type dihydrofolate reductase on Sephadex G-100.

The behavior of enzyme reducing folate-H_2 in the ame^r-3 mutant preparation on G-100 was markedly different (Fig. 1B). Two separate components in crude preparations were evident. Most of the enzyme exists in a form excluded ($V/V_0 = 1$) along with the large bulk proteins. In addition, a small portion (10-20%) has an elution volume, and ratio V/V_0, identical to the wild-type enzyme. Varying the size of the sample put on the column or reducing the amount of enzyme in the crude preparation by further genetic modification of this mutant had no effect on these results (Sirotnak and Hutchison, unpublished

data). When mutant enzyme, purified 6-8 fold by precipitation in 30% saturated ammonium sulfate, was added to the G-100 column, only the excluded form was observed (Fig. 1C). The result is the same when excluded enzyme collected from a previous run is put back on the same column. If the G-100 column is loaded with a mixture of wild-type and partially purified mutant preparations, enzyme comes off the column in two separate fractions (Fig. 1D) at elution volumes characteristic of each type. A mixture of crude material from both wild-type and mutant strains gave essentially the same result. In both cases, the recovery of enzyme characteristic for each preparation was equal to that amount put on the column. The same portion of *ame*^{r}-3 mutant enzyme in crude preparations was also excluded on G-200 and P-300 gel columns (Table 1). Note, at the same time, the increasing values (V/V_o) for the non

Table 1. The Elution of Mutant Dihydrofolate Reductase From Gel Columns With Different Exclusion Limits.

	V/V_o	
Gel	First Component	Second Component
G-100	1.0	2.0
G-200	1.0	2.7
P-300	1.0	3.0

excluded form.

Although the *ame*^{r}-3 mutation has, to some extent, been genetically (Sirotnak, Lunt and Hutchison, 1964) and enzymologically (Sirotnak, Donati and Hutchison, 1964) characterized, reasons for the differences seen here are not yet apparent. Genetic tests have rendered as unlikely the presence of an additional mutation in this strain. Moreover, other strains, derived from the *ame*^{r}-3 mutant by a transformation

of the wild-type under conditions favoring the selection of transformants bearing only a single mutation, exhibit identical enzymological properties.

Genetically determined structural changes, when altering the geometry of the enzyme molecule, might be expected to have some effect on elution volume. However, it would be difficult to reconcile the observed differences on this basis alone. On the other hand, if we attempt an explanation solely in terms of a molecular weight change, we must account for, at least, a 15-fold increase. Consistent with what is already known of the genetic alteration in this mutant is the possibility that the excluded component occurs as a result of a structural change, which favors association among reductase molecules, or with other, perhaps larger, proteins. Of additional interest, is the fact that mutant and wild-type enzymes exhibit identical elution patterns during DEAE chromatography (Sirotnak and Hutchison, unpublished results). This would lend support to the idea that the excluded component is an association of reductase molecules. Current investigations in our laboratory concern themselves with the nature of the excluded enzyme component in this and related mutants.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the capable technical assistance of Mrs. Mary Ann Kelley and valuable discussions with Doctors A. M. Albrecht and Louis Kaplan during the course of this work.

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