Genetic Approaches to Determination of Enzyme Quaternary Structure*

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ABSTRACT: Hybridization of variants of an oligomeric protein can provide information about the number of subunits in the oligomer. Such hybridization studies have generally been done *in vitro* using either genetic variants or chemically modified enzyme. A disadvantage of *in vitro* hybridization is that it can only be applied to proteins which can be reversibly dissociated into subunits. This paper presents two methods of obtaining electrophoretic variants of bacterial enzymes. The variants are hybridized *in vivo*, by random association of newly synthesized subunits. This method is not restricted to proteins which are reversibly dissociable and avoids artifacts which can arise in the course of chemical modification of protein. The variant protein used is enzymatically active and thus is likely to be similar to wild-type protein. The method has been applied to two enzymes, histidinol dehydrogenase and 6-phosphogluconate dehydrogenase. Each of these enzymes seems to be composed of two subunits.

In the determination of the number of subunits in oligomeric enzymes, enzyme hybridization experiments have proven useful. This method depends on obtaining two electrophoretically distinct variants of a single subunit type and allowing these subunits to associate. This general method and enzymes to which it has been applied have been reviewed by Klotz *et al.* (1970). Use of chemically modified enzymes for hybridization has been described by Meighen and Schachman (1970).

To perform this hybridization *in vitro* conditions must be known which permit dissociation and reassociation of the subunits. By observing the number of electrophoretically different aggregates which form, one can determine the number of subunits in the aggregate; the number of identical subunits is one less than the number of enzyme species formed by random association of the electrophoretically distinct subunit types. It is convenient if the hybrid and parental enzyme types can be detected in the electrophoresis gel. For many enzymes, one or both of these conditions cannot be met. This note presents several genetic methods which permit both of the conditions to be met. These methods should be applicable to many bacterial enzymes. We have studied the subunit structure of 6-phosphogluconate dehydrogenase (GND)[1] and the histidine biosynthetic enzyme, histidinol dehydrogenase (HDH).

Materials and Methods

Bacterial Strains. All strains used are described in Table I. These strains are derived from *Salmonella typhimurium* strain LT2. Some of these strains carry an F′ episome which is derived from *Escherichia coli*. This episome carries wild-type *E. coli* alleles of the structural genes for the enzymes histidinol dehydrogenase (*hisD*$^+$) and 6-phosphogluconate dehydrogenase (*gnd*$^+$). The HIS$^+$ revertants of *hisD3018* (*hisD3018R5*, *hisD3018R43*) were selected by J. Yourno and were obtained from him.

Growth of Cells and Preparation of Extracts. Cells were grown on the E medium of Vogel and Bonner (1956) containing 0.5% glucose as a carbon source. When appropriate, the medium was supplemented with histidine to a final concentration of 0.1 mM. A 500-ml culture was harvested by centrifugation (20 min at 3000*g*). The cells were washed with 50 ml of 0.05 M Tris buffer (pH 7.5) and finally suspended in 10 ml of the same buffer. Cells were disrupted by sonic oscillation and the cell debris was removed by centrifugation (60 min at 30,000*g*). These cell extracts were stored at 4°. Protein was estimated by the biuret method (Gornall *et al.*, 1949).

Gel Electrophoresis and Enzyme Assays. Chemicals for making gels were obtained from Canalco Industrial Corp. The electrophoresis method used is the standard method as described in the Canalco Manual with the exception that no stacking gel was used. Sucrose was added to the sample to a final concentration of 20% and the sample was then layered directly onto the separating gel. Gels were run at pH 8.6.

A histological stain was used to determine the position of HDH and GND activity in the gel. The dye mix and assay conditions were as described by Ames *et al.* (1963). For assay of HDH, histidinol was added (10^{-3} M) and when GND was assayed 6-phosphogluconate (3×10^{-3} M) was added. The gels were immersed in the reaction mixture and incubated 10–30 min (for HDH) or 30–40 min (for GND) to allow color to develop. Gels were then rinsed and stored in 2% acetic acid at 4°.

Results

6-Phosphogluconate Dehydrogenase. Electrophoretically distinct forms of the GND enzyme are produced by the closely related enteric bacteria *E. coli* and *S. typhimurium*. These enzymes are presented in Figure 1 (gels 1 and 2). A *Salmonella* strain was constructed which carried two copies of the GND structural gene, the normal chromosomal copy (*Salmonella*)

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[1] Abbreviations used are: GND, 6-phosphogluconate dehydrogenase; HDH, histidinol dehydrogenase.

TABLE I: Strains Used.[a]

Strain No.	Genotype	Relevant Genes Carried and Source[b]
LT-2	Wild type	*gnd*(S) *hisD*(S)
TR381	*hisEI640*/F'*his*$^+$*gnd*$^+$	*gnd*(C) *hisD*(C), *hisD*(S)
TR382	*hisFAHBCDG644*/ F'*his*$^+$*gnd*$^+$	*gnd*(C) *gnd*(S) *hisD*(C)
TR1478	*hisI570 hisD3018*(R5) *his01242*/F'*his*$^+$*gnd*$^+$	*hisD*(C) *hisD*(S*)
TR1479	*hisI570 hisD3018*(R43) *his01242*/F'*his*$^+$*gnd*$^+$	*hisD*(C) *hisD*(S*)
TR1480	*hisI570 hisD3018*(R5) *his01242*	*hisD*(S*)
TR1481	*hisI570 hisD3018*(R43) *his01242*	*hisD*(S*)

[a] All strains listed are derived from *S. typhimurium* strain LT-2. The F'*his*$^+$*gnd*$^+$ episome is derived from *E. coli*. Deletion *his-640* destroys the structural gene for GND (*gnd*) as well as the *hisE* and *hisI* genes. Deletion *hisD644* destroys the HDH gene (*hisD*) as well as the *hisF,A,H,B,C*, and *G* genes, but does not affect the *gnd* gene. The mutation *his01242* is a operator regulatory mutation which causes derepression of the adjacent histidine operon. [b] C = *E. coli;* S = *S. typhimurium;* * = mutant gene producing an electrophoretically variant enzyme.

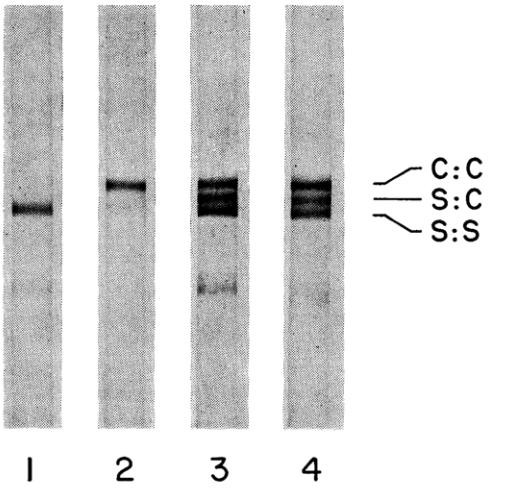

FIGURE 1: 6-Phosphogluconate dehydrogenase. Gel 1 contains an extract of wild-type *S. typhimurium* strain LT2. Gel 2 contains an extract of strain TR381, whose only functional gene for GND is derived from *E. coli*. Gels 3 and 4 are duplicates and contain extracts of the *Salmonella–E. coli* diploid strain TR382. Presumed subunit composition of each band is indicated at right (C = *E. coli* subunit; S = *S. typhimurium* subunit).

and a second copy carried by an F' episome derived from *E. coli*. Each of these genes functions in this strain and produces its particular form of the GND sunbunit. The enzymatically active oligomers are assembled *in vivo* from this mixed pool of subunits. When native enzyme from the merodiploid cell is subjected to gel electrophoresis, three species are apparent (Figure 1, gels 3 and 4). The fastest migrating species is indistinguishable from native *E. coli* enzyme (gel 1); the slowest band corresponds to native *Salmonella* enzyme (gel 2). The intermediate band is presumably a hybrid composed of subunits of both types. Since only one hybrid form appears, we conclude that GND is probably composed of two identical subunits. To our knowledge the subunit structure of this enzyme has not been investigated by other methods.

Histidinol Dehydrogenase. HDH from *Salmonella* and from *E. coli* are electrophoretically indistinguishable. Therefore mutant bacterial strains were used which produce active HDH which is electrophoretically distinct from wild-type enzyme. The strains used are HIS$^+$ revertants of a *hisD* (histidinol dehydrogenase) frame-shift mutant.

A frame-shift mutation causes the addition or deletion of one or more bases to the mRNA such that the reading phase of the message is altered and grossly abnormal protein is produced. Revertant strains can be selected which have regained the ability to form an active protein. Many such revertants are found to be normal except for a short sequence of adjacent improper amino acids (Streisinger *et al.*, 1966; Berger *et al.*, 1968; Yourno and Heath, 1969). Revertant proteins containing such a sequence of improper amino acids are likely to be electrophoretically distinguishable from wild-type proteins. For this reason, revertants of frame-shift mutations should be a rich source of electrophoretic variants. However, revertants of base substitution mutations could also be used as a potential source of electrophoretically variant enzyme.

Two revertants of frame-shift mutant *hisD3018* were used, *hisD3018R5* and *hisD3018R43*. Both produce HDH enzyme which is electrophoretically distinct from wild-type enzymes. Yourno and Heath (1969) have shown that HDH from *hisD3018R5* has a missense sequence of at least four amino acids. The amino acid sequence of HDH from *hisD3018R43* has not been investigated. Strains were constructed which contain the mutant *hisD* gene (*Salmonella*) in the chromosome and a wild-type *hisD* gene in an F' episome. The enzyme from these diploids was subjected to gel electrophoresis. Results using revertant R5 are presented in Figure 2.

In Figure 2, gel 1 contains *E. coli* HDH. Gel 2 contains enzyme produced by partially diploid strains having a wild-type *Salmonella* gene and a wild-type *E. coli* gene; the two enzymes are not resolved. Gel 3 contains the slower moving R5 mutant HDH. Gel 4 contains a mixture of wild-type HDH and R5 HDH; the two are easily resolved. Gel 5 contains enzyme produced by a single strain having both the R5 *hisD* gene and a wild-type *E. coli hisD*$^+$ gene. One hybrid species appears in addition to the two parental types.

Results using a second revertant *hisD3018R43* are presented in Figure 3. The mutant (R43) and wild-type enzymes are clearly resolvable. When mutant and wild-type subunits are synthesized and aggregate in the same cell, three forms of enzyme are produced. One form is electrophoretically like wild-type enzyme; another is like R43 enzyme. The third form has an intermediate electrophoretic mobility and acts like a hybrid enzyme having one wild-type and one mutant subunit. These results would be expected if HDH were composed of two identical subunits.

Complementation studies of the HDH structural gene *hisD* have revealed two complementation groups (Loper *et al.*, 1964). These results suggest that complementation

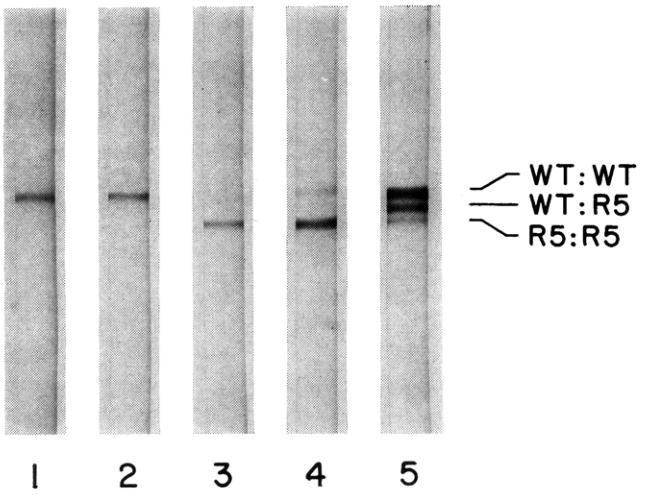

FIGURE 2: Histidinol dehydrogenase. Gel 1 contains extract of strain TR382 whose only *hisD* gene derived from *E. coli*. Gel 2 contains an extract of strain TR381 which has one wild-type *hisD* gene from *E. coli* and one from *S. typhimurium*. Gel 3 contains an extract of strain TR1480 which carries a mutant *Salmonella hisD* gene, *hisD3018R5*. Gel 4 contains a mixture of extracts of strain LT-2 (*S. typhimurium*) and of strain TR1480. Gel 5 contains an extract of strain TR1479 which carries a wild-type *E. coli hisD* gene and a mutant *S. typhimurium hisD* gene (*hisD3018R5*). Presumed subunit composition of each band is indicated at right (WT = wild type; R5 = mutationally altered subunits).

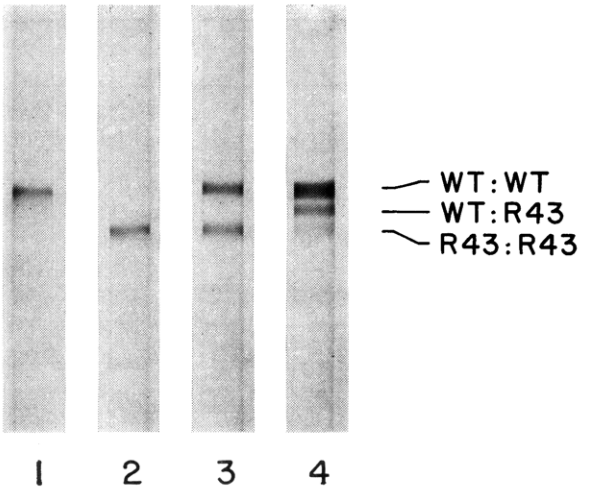

FIGURE 3: Histidinol dehydrogenase. Gel 1 contains an extract of wild-type *S. typhimurium* strain LT-2. Gel 2 contains an extract of strain TR1481 which carries a mutant *hisD* gene (*hisD3018R43*). Gel 3 contains a mixture of extracts of strain LT-2 and of strain TR1481. Gel 4 contains an extract of the partially diploid strain TR1478 which carries a wild-type *E. coli hisD* gene and a mutant *S. typhimurium hisD* gene (*hisD3018R43*). Presumed subunit composition of each band is indicated at right (WT = wild type; R43 = mutationally altered subunit).

between mutants in the two groups is due to intragenic complementation. However one could interpret these earlier data as indicating the existence of two genes and therefore dissimilar HDH subunits. If the enzyme had been composed of dissimilar subunits, no hybrid band would have appeared. Loper (1968) and Yourno (1968) have studied the molecular weight and composition of native HDH and HDH subunits. They conclude that the native enzyme is composed of two identical subunits. The hybridization behavior of HDH is consistent with their conclusion and indicates the existence of two identical subunits.

Discussion

Results presented here demonstrate two simple means of obtaining electrophoretic variants. One method makes use of interspecies hybrids. *S. typhimurium* and *E. coli* are advantageous for this purpose since their DNA base sequence homology is considerably less than perfect (McCarthy and Bolton, 1963; Schildkraut *et al.*, 1961). One might expect that these sequence differences would be reflected in differences in amino acid sequences of enzyme from these bacteria. The tryptophan synthetases of *S. typhimurium* and *E. coli* have been shown to differ extensively (Creighton *et al.*, 1966). In spite of the differences in base sequence between these organisms, their chromosomal maps are virtually identical and DNA is readily transferred from one species to the other (Sanderson, 1970; Taylor, 1970). Many F′ episomes have been constructed which carry a small part of the *E. coli* chromosome (Scaife, 1967; Low, 1968). Most of the *E. coli* chromosome is represented on one or more of these F′ episomes. Since all of these F′ episomes can be transferred into *S. typhimurium*, one can construct partially diploid hybrid strains, such as those used here, for almost any bacterial gene.

Another means of obtaining electrophoretic variants is through reversion of a mutation which causes complete loss of active enzyme. Since proteins produced by revertants of frame-shift mutants often have a sequence of several altered amino acids, many of these revertant proteins should differ electrophoretically from the wild type. This method could be applied to any enzyme if one can isolate frame-shift mutants of the enzyme's structural gene. Thus any enzyme could be studied which is dispensable to the cell under a particular set of growth conditions. The existence of a mutagen (ICR-191), which induces almost exclusively frame-shift mutations in bacteria, further simplifies this procedure (Ames and Whitfield, 1966; Berger *et al.*, 1968; Yourno and Heath, 1969). The revertants are selected because they form a functional (although not necessarily perfect) form of the enzyme. Thus the variant enzyme is not grossly atypical. Electrophoretic protein variants obtained in this way could, of course, also be used for *in vitro* hybridization studies.

There are several advantages to performing enzyme hybridization *in vivo* using genetically variant subunits. (1) Each of the two subunit variants is synthesized *in situ* and contributes to the subunit pool from which oligomers form. Therefore there is no need to obtain free subunits from some previously formed oligomer. This makes the method applicable to enzymes whose subunits are not reversibly separable. In addition, artifacts are avoided which might arise due to the rigors of subunit separation (*e.g.*, breakage of peptide bonds, irreversible denaturation). (2) The subunit variants used resemble the wild-type enzyme closely, since they are selected as being enzymatically active and are presumably able to associate normally. In contrast, formation of subunit variants by chemical modification may sometimes have drastic consequences for the subunit structure; loss of activity and abnormal aggregation properties may result (Meighen and Schachman, 1970). (3) By using genetic variants, one can generally be sure that each of the two subunit types is homogeneous. This homogeneity may be difficult to attain in chemically modified subunit popula-

tions, which may be heterogeneous in the numbers and types of derivatized groups per protein molecule.

The conclusions from *in vivo* hybridization studies might be misleading if subunit aggregation started with nascent rather than completed subunits. If this occurred there might be a tendency of subunits to aggregate primarily with other subunits synthesized on the same messenger molecule. Thus dissimilar subunit types, synthesized on different messages, might be prevented from forming hybrid enzyme. Although this possibility should be kept in mind, we know of no case in which this situation has been demonstrated.

Results such as those presented here which depend on enzymatic activity might be misinterpreted if one or more of the hybrid oligomers is highly unstable or enzymatically inactive. In these cases one or more of the hybrid types would be missed and an incorrect subunit number concluded. Use of several different variant enzyme types mitigates but does not completely eliminate this difficulty. Interpretation of results for enzymes with dissimilar subunits is somewhat more complicated and might necessitate use of mutant forms of both subunits. However, study of such enzymes could be approached with methods such as those presented here. Because the results presented here do not eliminate the above possibilities, our conclusions as to subunit number should be taken as tentative.

Genetic techniques have been used to obtain information about subunit structure of several enzymes in various organisms. One of the earliest users of this method is Schwartz (1960), who studied an esterase from maize. A more recent example is the study of octanol dehydrogenase (Pipkin, 1969). Additional examples are described in a volume on isozymes (Vesell, 1968). Methods such as those presented above should make a genetic approach to the study of enzyme subunit structure more widely applicable.

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