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Mutant Analysis of Glyceraldehyde 3-Phosphate Dehydrogenase in *Escherichia coli*

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NAD^+-specific glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from *Escherichia coli* was purified to homogeneity by a relatively simple procedure involving affinity chromatography on agarose–hexane–NAD^+ and repeated crystallization. Rabbit antiserum directed against this protein produced one precipitin line in double-diffusion studies against the pure enzyme, and two lines against crude extracts of wild-type *E. coli* strains. Both precipitin lines represent the interaction of antibody with determinants specific for glyceraldehyde 3-phosphate dehydrogenase. Nine independent mutants of *E. coli* lacking glyceraldehyde 3-phosphate dehydrogenase activity all possessed some antigenic cross-reacting material to the wild-type enzyme. The mutants could be divided into three groups on the basis of the types and amounts of precipitin lines observed in double-diffusion experiments; one group formed little cross-reacting material. The cross-reacting material in crude cell-free extracts of several of the mutant strains were also tested for alterations in their affinity for NAD^+ and their phosphorylative activity. The cumulative data indicate that the protein in several of the mutant strains is severely altered, and thus that glyceraldehyde 3-phosphate dehydrogenase is unlikely to have an essential, non-catalytic function such as buffering nicotinamide nucleotide or glycolytic-intermediate concentrations. Others of the mutants tested have cross-reacting material which behaved like the wild-type enzyme for the several parameters studied; the proteins from these strains, once purified, might serve as useful analogues of the wild-type enzyme.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) has several unusual properties that have made it the object of extensive investigation since its initial purification from yeast by Warburg & Christian (1939). In particular, although the enzyme isolated from a variety of sources has been shown to be a tetrameric protein composed of identical subunits (D'Alessio & Jossi, 1971*b*; Harrington & Karr, 1965; Harris & Perham, 1965), there appear to be only two active sites per molecule (Krimsky & Racker, 1963; MacQuarrie & Bernhard, 1971; Malhotra & Bernhard, 1968). In addition to this so-called half-of-the-sites reactivity, the enzyme isolated from rabbit muscle also displays the phenomenon of negative co-operativity in which successive molecules of the cofactor, NAD^+, bind with decreasing affinity (Conway & Koshland, 1968; Devijlder & Slater, 1968). Also, it has been generally observed that glyceraldehyde 3-phosphate dehydrogenase constitutes an extraordinarily high proportion of the cell's soluble protein (approx. 2–10%; see Cori *et al.*, 1948; Allison & Kaplan, 1964; D'Alessio & Jossi, 1971*a*) and that much of the enzyme apparently exists *in situ* as the 3-phosphoglyceroyl derivative (Bloch *et al.*, 1971).

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To date, little agreement has been reached in trying to explain the molecular basis of these observed phenomena or their importance with respect to the cell's metabolism. We previously reported the isolation of mutants of *Escherichia coli* lacking glyceraldehyde 3-phosphate dehydrogenase activity (Hillman & Fraenkel, 1975). Data were presented which indicated that the genetic defects present in nine independently isolated strains probably involved mutations in a single genetic locus called *gap*. It was also shown with a temperature-sensitive mutant that *gap* is probably the structural gene for the enzyme. In the present paper I report studies in which crude extracts of the mutants were surveyed by immunological methods for the presence of glyceraldehyde 3-phosphate dehydrogenase-like proteins which could serve as useful analogues of the wild-type enzyme. In addition, several catalytic properties of the enzyme were examined in parent and mutant crude extracts to help to assess the extent of the protein alteration in the mutant strains.

Materials and Methods

Materials

Unless otherwise specified, all reagents used in this study were obtained from Sigma Chemical Co.,

St. Louis, MO, U.S.A., and were the highest grade available. Purified enzymes used in this study were from Boehringer–Mannheim Corp., New York, NY, U.S.A. D-Threose 2,4-bisphosphate was prepared by the methods of Racker *et al.* (1959).

Media and bacterial strains

Media, and the isolation and growth properties of the *gap* mutants listed in Table 3, have been previously reported (Hillman & Fraenkel, 1975). Cultures of parent and mutant strains were grown in M63 medium supplemented with malate (25mM), glycerol (12.5mM), thiamin (vitamin B_1; 1μg/ml) and Difco (Detroit, MI, U.S.A.) Casamino acids (0.25%, w/v). Cultures were routinely tested for revertants by spreading 0.1ml samples on M63 agar containing 0.4% (w/v) glucose.

Enzyme assays

Crude cell-free extracts were prepared from 25–100ml cultures as previously described (Hillman & Fraenkel, 1975) except that 20mM-Tris/HCl, pH8.0, containing 2mM-2-mercaptoethanol and 2mM-EDTA served as buffer for sonication. Glyceraldehyde 3-phosphate dehydrogenase catalytic activity was measured by the oxidative-arsenolysis assay described by Allison & Kaplan (1964), as modified by D'Alessio & Jossi (1971*a*). Glyceraldehyde 3-phosphate dehydrogenase phosphorylative activity was measured by the $[^{32}P]P_i$–$[\gamma\text{-}^{32}P]ATP$ exchange assay described by D'Alessio & Jossi (1971*a*), with the following modifications: 3mM-3-phosphoglyceric acid was substituted for 2-phosphoglyceric acid and phosphoglyceromutase, and 1μg of phosphoglycerate kinase (from rabbit muscle) was included in the reaction mixture. The reaction was initiated by the addition of 10μl of crude, cell-free extract.

Purification of wild-type enzyme

All of the following operations were performed at 4°C unless otherwise specified. Buffer refers to 20mM-Tris/HCl (adjusted to pH7.7 or 8.0 at 5°C with Tris base) containing 2mM-EDTA and 2mM-2-mercaptoethanol. Centrifugations were performed at 15000*g* in a Sorvall model RC2-B refrigerated centrifuge. Steps 3, 4, 5 and 8 were adapted from the methods of D'Alessio & Jossi (1971*a*,*b*).

Step 1: *growth and harvesting of cells.* A 16-litre batch culture of *E. coli* strain K10 was grown with aeration at 37°C to stationary phase in glucose minimal medium supplemented with thiamin as previously described (Hillman & Fraenkel, 1975). The cells were harvested by centrifugation for 30min and stored at −20°C until used.

Step 2: *crude extract.* Packed cells (20g wet wt.) were uniformly suspended in 20ml of buffer, pH8.0, and treated with a Biosonik II ultrasonicator (Bronwill Scientific Co., Rochester, NY, U.S.A.) in 15s bursts for a total of 5min. A salt/ice bath was used to prevent the temperature of the extract from rising above 7°C. Unbroken cells and cell debris were removed by centrifugation for 30min and the pellet was washed once by resuspension in 50ml of buffer. The combined supernatants were diluted with buffer to 106ml total volume (fraction I).

Step 3: *streptomycin precipitation.* Streptomycin sulphate (1.38g) dissolved in 27.8ml of buffer, pH8.0, was added to fraction I with stirring. After 15min the resulting precipitate was removed by centrifugation for 30min and the supernatant diluted to 132ml with buffer (fraction II).

Step 4: *65–95%-satd.-ammonium sulphate fractionation.* Solid $(NH_4)_2SO_4$ (53.4g; enzyme grade; Schwarz–Mann, Orangeburg, NJ, U.S.A.) was dissolved with stirring in fraction II. After 10min incubation, the resulting precipitate was removed by centrifugation for 30min and the supernatant (152ml) treated by dissolving an additional 31.1g of solid $(NH_4)_2SO_4$. After standing overnight at 4°C the precipitate was recovered by centrifugation for 30min and taken up in 4ml of buffer (fraction III).

Step 5: *charcoal treatment.* Fraction III was exhaustively dialysed against buffer, pH7.7. Acid-washed Norit A (300mg; Smith & Khorana, 1963) was then added and kept in suspension by gentle stirring. After 2h the Norit was removed by centrifugation for 30min and washed once with 11.25ml of buffer, pH7.7. The combined supernatants were clarified with a final centrifugation for 30min (fraction IV).

Step 6: *affinity chromatography.* A 1.5cm×40cm column was packed with 30ml of agarose–hexane–NAD^+ type 1 (P-L Biochemicals, Milwaukee, WI, U.S.A.) and equilibrated with 250ml of buffer, pH7.7, at 4°C. Fraction IV was applied and the column was washed with 3 bed vol. of buffer at a flow rate of 1ml/min. A linear gradient of 0–0.5mM-NAD^+ in buffer, pH7.7 (total volume 120ml) was used to elute the enzyme, and 5ml fractions were collected. Those fractions with most of the eluted glyceraldehyde 3-phosphate dehydrogenase activity were pooled and the protein was concentrated by saturating the sample with solid $(NH_4)_2SO_4$. After standing overnight at 4°C the precipitate was recovered by centrifugation for 30min and taken up in 1ml of buffer, pH8.0 (fraction V).

Step 7: *crystallization.* Protein in fraction V was loaded with NAD^+ by the addition of 4.3mg of solid NAD^+ (10mol/144000g of protein). After incubation for 30min, neutral saturated $(NH_4)_2SO_4$ was added in 50μl amounts until a faint turbidity was observable. After incubation overnight on ice, crystals that had formed were recovered by centrifugation, and the crystallization procedure was repeated (fraction VI).

The purified protein so obtained was stored as crystals in 2 M-$(NH_4)_2SO_4$ at 4°C. Before use, samples of fraction VI were generally made 0.03 M in 2-mercaptoethanol and incubated at room temperature for 30min by the methods of D'Alessio & Jossi (1971*b*). $(NH_4)_2SO_4$ was then removed by dialysis against buffer, pH 8.0.

Gel electrophoresis

Native and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the methods of Davis (1964) and Weber & Osborn (1969), respectively. Preparation of antigen–antibody complexes for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis followed the methods of Roberts & Roberts (1975). The stain for glyceraldehyde 3-phosphate dehydrogenase activity on native gels was described by Brown & Wittenberger (1971).

Immunological methods

Antiserum to purified glyceraldehyde 3-phosphate dehydrogenase from *E. coli* K10 was prepared in rabbits by injecting 400 μg of protein from fraction VI emulsified with complete Freund's adjuvant both intramuscularly and intradermally. The immunization was repeated on days 14 and 21, and on day 28 samples of peripheral blood were collected. The serum was stored at 4°C with 0.01% NaN_3 as preservative. Antibody titre was determined by quantitative precipitin assays by using fraction VI or crude cell-free extract of *E. coli* K10 as the source of antigen. The amount of protein in the precipitates was determined by the method of Lowry *et al.* (1951) by using bovine serum albumin as standard. Double-diffusion (Ouchterlony) analyses of purified enzyme in fraction VI and of parent and mutant cell-free extracts used agar immuno-plates (Hyland Corp., Costa Mesa, CA, U.S.A.).

Results

Antigenic properties of cell-free extracts

The earlier finding (Hillman & Fraenkel, 1975) that all nine independent *gap* mutants could be reverted to growth on glucose and that such revertants had regained some or all of the parental glyceraldehyde 3-phosphate dehydrogenase activity suggested that the various genetic lesions being studied were point mutations. Thus, with the possible exception of mutant DF221, which had been shown to possess an amber mutation in the *gap* gene, all of the mutant strains were expected to possess a modified but potentially useful analogue of glyceraldehyde 3-phosphate dehydrogenase. This prediction was tested by screening crude extracts of the mutant strains for antigenic cross-reacting material with antiserum prepared against wild-type *E. coli* glyceraldehyde 3-phosphate dehydrogenase. The enzyme used in preparing the antiserum was purified from strain K10 as described in the Materials and Methods section and summarized in Table 1. Briefly, the procedure involved several initial steps (1–4) designed to concentrate and partially to purify the enzyme. In step 6 the sample, which had been treated with activated charcoal to remove NAD^+ bound to glyceraldehyde 3-phosphate dehydrogenase, was applied to an agarose–hexane–NAD^+ column and the enzyme was eluted with a linear gradient of NAD^+. The profiles for activity, protein (A_{280}) and NAD^+ (A_{260}) in the column fractions from this step are shown in Fig. 1. The three fractions containing approx. 85% of the eluted activity were pooled and the protein was concentrated. At this stage, electrophoresis in native gels indicated the presence of small amounts of contaminating proteins; these were eliminated by repeated crystallization of the enzyme in the presence of excess NAD^+. Although there was a significant loss in the total activity (about 25%) as a result of the two crystallization steps, the resulting fraction VI

Table 1. *Summary of purification*

The individual steps are described in the Materials and Methods section. Affinity chromatography was on agarose–hexane–NAD^+ type 1 (P-L Biochemicals). Glyceraldehyde 3-phosphate dehydrogenase activity was measured by using the oxidative-arsenolysis assay, with glyceraldehyde 3-phosphate as substrate. Specific activities are units (μmol of product formed/min) per mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Fraction	Step	Protein (mg/ml)	Specific activity (units/mg of protein)	Total activity (units)
I	Extract	11.1	0.9	1090
II	Streptomycin	2.8	2.9	1103
III	65–95%-satd.-$(NH_4)_2SO_4$	22.0	8.2	1044
IV	Norit A	4.8	10.0	858
V	Affinity chromatography	9.9	30.0	416*
VI	Crystallization	3.8	44.6	335

* Represents 85% of the total activity eluted from the column.

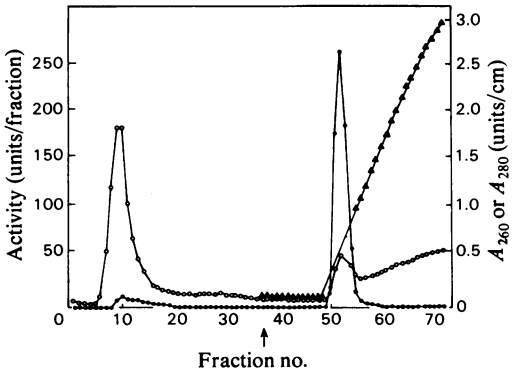


Fig. 1. *Purification of E. coli glyceraldehyde 3-phosphate dehydrogenase by affinity chromatography*
Fractions (5ml) eluted from the agarose–hexane–NAD^+ column were analysed for their enzyme activity (●) by using the arsenolysis assay described in the Materials and Methods section. In addition, the A_{260} (▲) and A_{280} (○) was determined for each fraction as a measure of NAD^+ and protein content, respectively. The NAD^+ gradient used to elute glyceraldehyde 3-phosphate dehydrogenase was initiated at the point indicated by the arrow.

appeared essentially pure when examined by electrophoresis in native and sodium dodecyl sulphate/polyacrylamide gels (Fig. 2). The subunit molecular weight of the purified *E. coli* glyceraldehyde 3-phosphate dehydrogenase was roughly determined by comparing its migration on sodium dodecyl sulphate/polyacrylamide gels with that of proteins with known subunit molecular weights. The results obtained (Fig. 2) indicate a value of 35000–36000, in agreement with the value obtained by D'Alessio & Jossi (1971*b*) using high-speed sedimentation-equilibrium techniques.

When samples of fraction VI were used to immunize a rabbit, an antiserum was obtained which precipitated the enzyme but did not inhibit catalytic activity. Quantitative precipitin assays using fraction VI or crude extracts of *E. coli* K10 indicated that the maximum amount of precipitate (equivalence point) occurred when samples containing 5–10 units (μmol of product formed/min) of glyceraldehyde 3-phosphate dehydrogenase activity/ml were incubated with equal volumes of undiluted serum. In subsequent experiments wild-type crude extracts were adjusted to this activity by dilution with buffer, and mutant extracts were adjusted to the equivalent protein concentration (2mg/ml).

The specificity of the antiserum was tested by using the double-diffusion technique: with pure protein (fraction VI) as antigen a single, sharp precipitin line was observed (Fig. 3). By contrast, with crude extracts of *E. coli* K10 or the other parental strains,

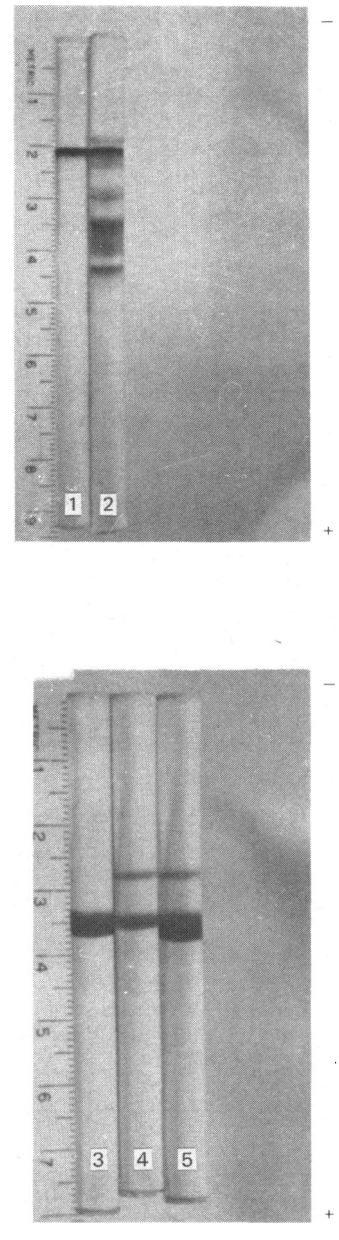


Fig. 2. *Native and sodium dodecyl sulphate/polyacrylamide gels of purified glyceraldehyde 3-phosphate dehydrogenase*
Gels 1 and 2 are 7.5% polyacrylamide gels, containing approx. 100μg of fractions VI and IV, respectively. Gels 3, 4 and 5 are sodium dodecyl sulphate/polyacrylamide gels which contained: gel 3, 50μg of fraction VI; gel 4, 5μg of rabbit muscle creatine kinase (40000-dalton subunit) and 10μg of rabbit muscle lactate dehydrogenase (36000-dalton subunit); gel 5, 50μg of fraction VI, 5μg of creatine kinase and 10μg of lactate dehydrogenase. Migration was from top to bottom.

two precipitin lines were generally visible. Several lines of evidence suggested that both precipitin lines represent interaction of antibody with antigenic determinants specific for glyceraldehyde 3-phosphate dehydrogenase. First, when crude extracts of the parent strain were tested in double-diffusion plates next to samples of fraction VI, spurs of non-identity were not observed for either of the precipitin lines (Fig. 3). Second, when antiserum was absorbed with purified protein from fraction VI, and then reacted in double-diffusion plates against crude extracts of K10, both precipitin lines were eliminated (Fig. 4). Third, when mutant DF234 (*gap*-7), a temperature-sensitive *gap* mutant, was grown at 26°C and cell-free extracts were tested by double diffusion, two precipitin lines homologous with the two lines produced by its parent were observed (Fig. 5). However, when the same experiment was done with cells grown at 40°C both precipitin lines were grossly distorted, in contrast with the parental pattern, which was unaltered.

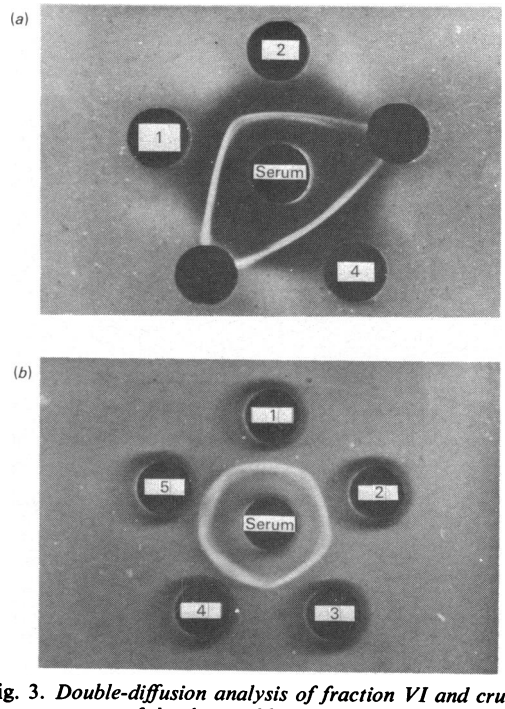


Fig. 3. *Double-diffusion analysis of fraction VI and crude extracts of the three wild-type parental strains*
(*a*) Antiserum (centre well) was made to react in double-diffusion plates against a sample of fraction VI (well no. 1) and crude extracts of strain K10 (wells nos. 2 and 4), as described in the Materials and Methods section. (*b*) Antiserum (centre well) was made to react against crude extracts of strains K10 (well no. 1), DF230 (wells nos. 2 and 3) and JC4110 (wells nos. 4 and 5).

Thus crude extracts of wild-type strains probably contain two forms of glyceraldehyde 3-phosphate dehydrogenase. The nature of these two forms has not yet been determined, but seems not to involve

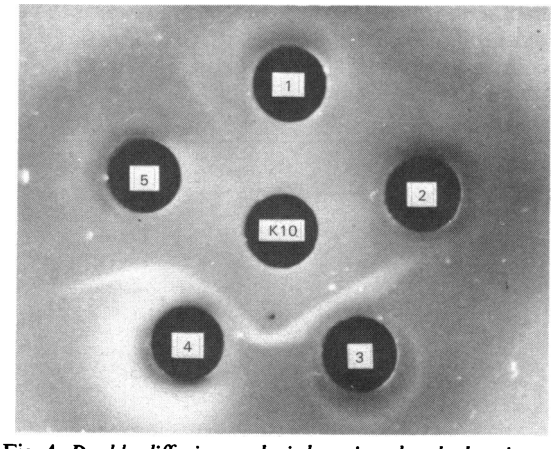


Fig. 4. *Double-diffusion analysis by using absorbed antisera*
Samples (0.1ml) of antiserum were mixed and incubated overnight with: 1, 38μg of protein from fraction VI; 2, 3.8μg of protein from fraction VI; 3, Tris/HCl control; 4, 0.1ml of goat anti-(rabbit γ-globulin) (1:10000, Antibodies Inc., Davis, CA, U.S.A.); 5, 0.1ml of *E. coli* K10 extract (3.0mg of protein/ml). After removal of the resulting precipitates by centrifugation (15000*g* for 30min) samples of the absorbed antiserum were made to react in double-diffusion plates against crude extract of wild-type strain K10 (centre well).

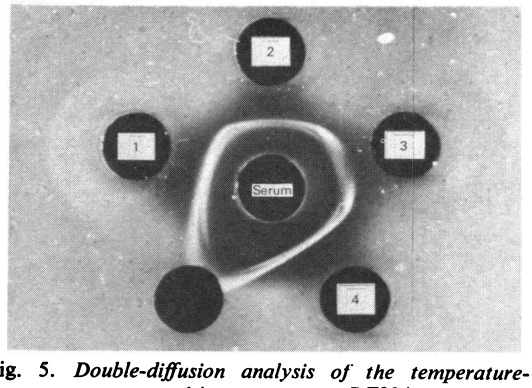


Fig. 5. *Double-diffusion analysis of the temperature-sensitive gap mutant, DF*234
Crude extracts of strain DF234, and the wild-type strain, K10, grown in glycerol-supplemented malate media, were made to react against antiserum (centre well) in double-diffusion plates, as described in the Materials and Methods section. Wells 1 and 3 contained extract of strain DF234 grown at 26 and 40°C, respectively, and wells 2 and 4 contained extracts of strain K10 grown at 26 and 40°C, respectively.

differences in the amount of bound ligands: treatment of crude extracts with charcoal or arsenate to discharge, respectively, bound NAD^+ or acyl groups did not affect the double-diffusion pattern obtained. Preliminary evidence also tended to exclude the possibility that the inner precipitin line represented interaction of antibody with a degradation product of the native enzyme: when the antigen–antibody complexes formed on incubating antiserum with crude extracts of strain *E. coli* K10 were tested by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels, the pattern of protein bands obtained was identical with the pattern obtained when samples of fraction VI served as the source of antigen. Had the crude extract contained significant amounts of a degradation product, then an additional band, migrating faster than the wild-type subunit, should have been observed on these gels if it did not overlap with the band corresponding to the light chain of immunoglobulin G. The likeliest explanation for the

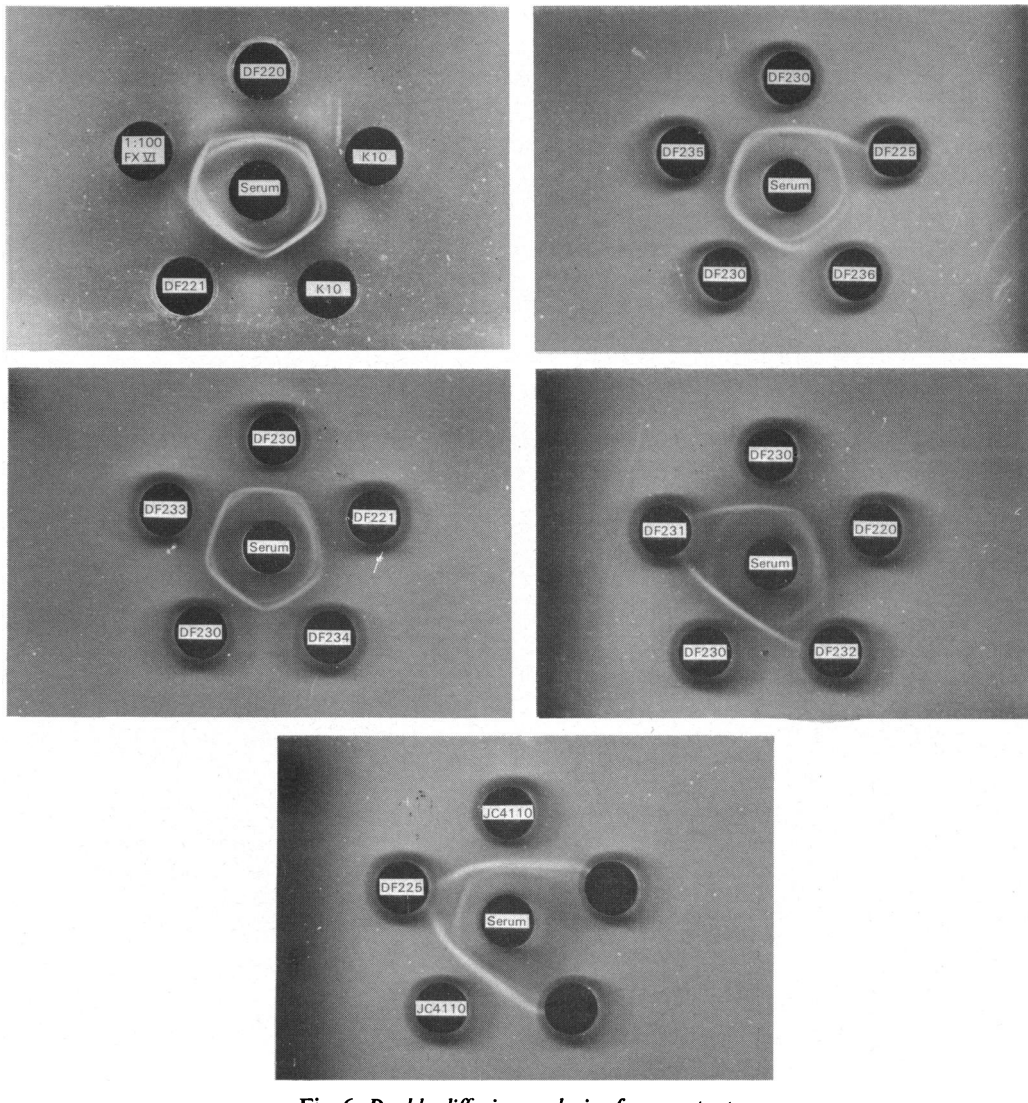


Fig. 6. *Double-diffusion analysis of gap mutants*

Crude extracts were prepared from cultures of the nine independent *gap* mutants and their parents, grown in glycerol-supplemented malate media at 30°C. Samples of the extracts were made to react against antiserum in double-diffusion plates as previously described. Abbreviation: FX VI, fraction VI.

occurrence of two precipitin lines when crude extracts of the wild-type strains are tested in double-diffusion plates would be that glyceraldehyde 3-phosphate dehydrogenase in *E. coli* exists in a dimer–tetramer equilibrium, as has been shown to occur with the pig muscle enzyme (Ovadi *et al.*, 1971). However, direct evidence to substantiate this hypothesis has not yet been obtained.

Once the specificity of the antiserum for determinants of glyceraldehyde 3-phosphate dehydrogenase had been established, it was possible to screen the nine independent *gap* mutants for the presence or absence of an antigenically related protein (Fig. 6). The nine mutants could be divided into three classes on the basis of the double-diffusion patterns that they generated. The first class, which included strains DR233 (*gap*-6) and DF235 (*gap*-8), appeared wild-type in having much more of the outer precipitin line than the inner one. The second class, composed of strains DF225 (*gap*-3), DF231 (*gap*-4) and DF232 (*gap*-5), appeared to possess only trace amounts of a precipitin line which fused with the faint inner line produced by the parental extracts. Strains DF220 (*gap*-1), DF221 (*gap*-2), DF234 (*gap*-7) and DF236 (*gap*-9) constituted a third class intermediate in pattern between the first two; both precipitin lines were present, but, quantitatively, there seemed to be less of the outer line and more of the inner line than was seen in the parental pattern.

Other properties of mutant cross-reacting material

Certain properties normally associated with wild-type glyceraldehyde 3-phosphate dehydrogenase could be surveyed in crude extracts of the mutant strains without additional purification. One such property, the high affinity of the enzyme for NAD^+, was assessed indirectly by determining whether or not mutant cross-reacting material could bind to agarose–hexane–NAD^+. Cell-free extracts (1 ml) of the *gap* mutants DF220, DF225, DF231, DF234 and DF235 and their parents were dialysed overnight and treated with 10 mg of activated Norit A for 1 h over ice. After removal of the charcoal by centrifugation (15000*g* for 20 min), the entire amount was placed on a 1 ml column of agarose–hexane–NAD^+ previously equilibrated with buffer. The columns were washed with 8 bed vol. of buffer and then bound protein was eluted with 0.5 ml of 10 mM-NAD^+ in buffer. Fractions of the eluate (1 ml) were collected and screened for cross-reacting material and, in the case of the parents, for enzyme activity. Of the five mutant strains surveyed, only DF235 (*gap*-8) behaved in a fashion identical with the parental strains in that its cross-reacting material both bound to the agarose–hexane–NAD^+ and was eluted by 10 mM-NAD^+ to give a single, sharp precipitin line. The cross-reacting material from the other four mutant strains was either lost in the wash fractions or bound irreversibly to the agarose–hexane–NAD^+.

A second parameter of glyceraldehyde 3-phosphate dehydrogenase that could be studied directly in crude extracts of the mutant strains was the catalytic integrity of the phosphorylative head of the enzyme. Three mutants, DF220, DF221 and DF225, and their parents, were tested for their ability to catalyse the phosphate-exchange reaction between orthophosphate and 1,3-bisphosphoglycerate (Table 2). In this assay 1,3-bisphosphoglycerate is generated from 3-phosphoglycerate and ATP by the enzyme phosphoglycerate kinase. In the absence of NADH, 1,3-bisphosphoglycerate reacts with the wild-type enzyme to form the stable acyl-enzyme complex, 3-phosphoglyceroyl-glyceraldehyde 3-phosphate dehydrogenase. Reversal of these reactions in the presence of $[^{32}P]P_i$ yields $[\gamma\text{-}^{32}P]ATP$. When samples of crude extracts from the wild-type strain K10 were tested in this system and the $[\gamma\text{-}^{32}P]ATP$ was recovered from the reaction mixture by absorption on activated charcoal, $[\gamma\text{-}^{32}P]ATP$ formation was indeed dependent on the presence of the substrates (3-phosphoglycerate and ATP) and glyceraldehyde 3-phosphate dehydrogenase in the crude extract. The dependence of activity on the dehydrogenase was also demonstrated by the addition of the specific inhibitor threose 2,4-bisphosphate (Racker *et al.*, 1959). Extracts of the three mutants, DF220, DF221 and DF225, gave only background ^{32}P incorporation in this assay.

Discussion

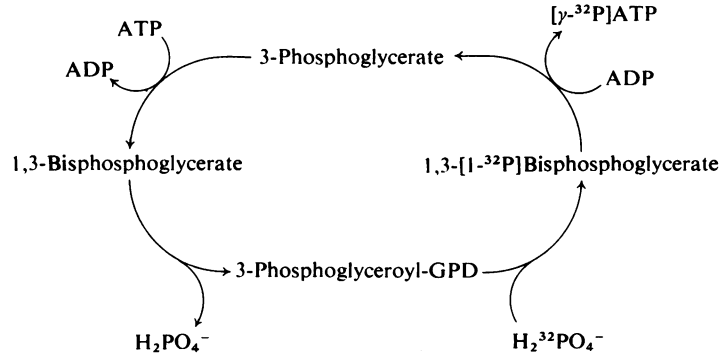
The high affinity of glyceraldehyde 3-phosphate dehydrogenase for its cofactor NAD^+ provided the rationale for purifying this enzyme from *E. coli* strain K10 by affinity chromatography to agarose–hexane–NAD^+. After repeated crystallization the purified product appeared identical with the enzyme isolated by other, less efficient, approaches (Allison & Kaplan, 1964; D'Alessio & Jossi, 1971*a*).

When samples of the purified enzyme were used to immunize a rabbit, a low-titre antiserum was obtained which reacted with *E. coli* glyceraldehyde 3-phosphate dehydrogenase to produce a precipitate, but which had no direct inhibitory effect on its catalytic activity. Similar findings have been observed previously (Elödi, 1958), and probably reflect the remarkable extent to which the primary structure of the active site has been conserved throughout phylogeny (Allison, 1968).

A survey of cross-reacting material in cell-free extracts of nine *gap* mutants permitted their distribution into three groups on the basis of inner and outer precipitin lines which formed in double-diffusion analysis. Mutants comprising one group appeared to have the normal, wild-type complement of both antigenic components responsible for forming the outer

Table 2. *[^{32}P]P_i–[γ-^{32}P]ATP exchange activity of three gap mutants*

The assay was performed as described in the Materials and Methods section. Abbreviation: GPD, glyceraldehyde phosphate dehydrogenase.



Strain	System	[γ-^{32}P]ATP recovered (c.p.m.)
K10 (parent)	Complete	2940
	–3-phosphoglycerate	137
	–ATP	218
	–extract	121
	Complete + 0.5 M-threose 2,4-bisphosphate	549
DF220 (*gap*-1)	Complete	146
DF221 (*gap*-2)	Complete	156
DF225 (*gap*-3)	Complete	158

and inner lines. A second class of mutants possessed only trace amounts of cross-reacting material, and appeared to lack entirely the antigenic component responsible for forming the major outer precipitin line. Finally, a third group of mutants was observed in which, on a semi-quantitative basis, the amount of outer precipitin line was decreased in proportion to the increase in the amount of inner precipitin line.

Of the five mutant strains tested for their ability to bind to and be eluted from agarose–hexane–NAD^+, only DF235 (*gap*-8), which gave a wild-type double-diffusion pattern, appeared to behave like the parental enzyme. The cross-reacting material in the other four mutants tested, whose double-diffusion patterns differed from the wild-type, clearly showed altered behaviour in this study, probably reflecting an alteration in their affinities for the coenzyme, NAD^+.

The inability of the three *gap* mutants tested to catalyse the phosphate-exchange reaction was expected on the basis of earlier work with the rabbit muscle enzyme. Although Krimsky & Racker (1963), using iodoacetic acid treatment, were able to eliminate completely the oxidative activity of the enzyme while only partially affecting the phosphorylative activity, subsequent studies (Harting & Velick, 1954; Park & Koshland, 1958; Murdoch & Koeppe, 1964) have shown bound NAD^+ to be an essential requirement for both catalytic functions. Although NAD^+ is not itself a reactant, its binding apparently effects a conformational change necessary for activity of the phosphorylative head. The observation that the cross-reacting material in mutants DF220, DF221 and DF225 possess altered affinity for NAD^+ suggests that this may be the cause of the inability of these strains to catalyse phosphate exchange. Alternatively, the phosphorylative heads of the enzymes in these strains may have been directly damaged by their respective mutations.

On the basis of its high concentration in the cell's cytoplasm and its curious affinity for NAD^+, it has been suggested that glyceraldehyde 3-phosphate dehydrogenase may serve some essential, non-catalytic function such as the buffering of nicotinamide nucleotide or glycolytic-intermediate concentrations (Bloch *et al.*, 1971; Conway & Koshland, 1968). The finding that all nine of the *gap* mutants under investigation possessed at least some cross-reacting material when tested with antiserum directed against the wild-type enzyme does not permit us definitively to discount this possibility. The data thus far accumulated, however, make it seem somewhat unlikely: as summarized in Table 3, extracts of several of the mutants studied, aside from being unable to catalyse the physiological interconversion of glyceraldehyde 3-

Table 3. *Summary of the properties of glyceraldehyde 3-phosphate dehydrogenase in nine mutants*
Activity *in vitro* was measured by the ability to catalyse both the oxidative phosphorylation of glyceraldehyde 3-phosphate and the reductive dephosphorylation of 1,3-bisphosphoglycerate (Hillman & Fraenkel, 1975). NAD^+ affinity was measured by the ability of cross-reacting material to bind to agarose–hexane–NAD^+ type 1, and phosphorylative activity was measured by the ability to catalyse the $[^{32}P]P_i$–$[\gamma\text{-}^{32}P]ATP$ exchange reaction.

Strain	*gap* allele	Activity *in vitro*	Double-diffusion pattern	NAD^+ affinity	Phosphorylative activity
DF220	1	None	Altered	Altered	None
DF221	2 (amber)	None	Altered	Altered	None
DF225	3	None	Altered	Altered	None
DF231	4	None	Altered	Altered	—
DF232	5	None	Altered	—	—
DF233	6	None	Normal	—	—
DF234	7 (ts)	Thermolabile	Altered	—	—
DF235	8	None	Normal	Normal	—
DF236	9	None	Altered	—	—

phosphate and 1,3-bisphosphoglycerate, show altered affinity for the coenzyme NAD^+, defective phosphorylative activity, and grossly altered serological cross-reactivity with antiserum directed against the wild-type glyceraldehyde 3-phosphate dehydrogenase. Other parameters associated with the wild-type enzyme, such as co-operativity in NAD^+ binding, subunit interactions and affinity for orthophosphate, could also be examined, but would require the partial or complete purification of the altered protein. In this regard, the purification procedure devised in this study appears to be directly applicable to the purification of the altered protein from at least two mutant strains (DF233 and DF235); other more conventional approaches may have to be used to purify the proteins from the other mutants.

Aside from shedding additional light on the possible essentiality of glyceraldehyde 3-phosphate dehydrogenase, the apparent cross-section of altered proteins obtainable from the mutant strains, once purified, would be likely to provide useful analogues of the wild-type enzyme to help to resolve the difficult issues of this enzyme's structure and function.

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