The Mechanism of 2-Keto-3-deoxy-6-phosphogluconate Aldolase

III. NATURE OF THE INACTIVATION BY FLUORODINITROBENZENE*

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SUMMARY

2-Keto-3-deoxy-6-phosphogluconate aldolase of Pseudomonas putida reacts with about 4 moles of 1-fluoro-2,4-dinitrobenzene per mole of aldolase; this is accompanied by 80 to 90% loss in activity. Both the arylation reaction and the inactivation are prevented by 2-keto-3-deoxy-6-phosphogluconate, 2-keto-3-deoxy-6-phosphogalactonate, and inorganic phosphate. The substrate binding site was then blocked by adding 2-keto-3-deoxy-6-phosphogalactonate and reducing the azomethine linkage to a lysine residue with sodium borohydride. Subsequent dialysis of the treated enzyme to remove noncovalently bound 2-keto-3-deoxy-6-phosphogalactonate, followed by reaction with fluorodinitrobenzene, results in only one of the four originally observed dinitrophenylations. This indicates that 3 molecules of fluorodinitrobenzene react in or close to the active site. Only the lysine ε-amino group is dinitrophenylated, and these lysines were shown to be distinct from those forming an azomethine with pyruvate, 2-keto-3-deoxy-6-phosphogalactonate, and, presumably, 2-keto-3-deoxy-6-phosphogluconate. The $K_m$ of dinitrophenylated aldolase for 2-keto-3-deoxy-6-phosphogluconate is about 2-fold higher than that of native aldolase, whereas there is a 10-fold decrease in $V_{max}$. Thus, the loss in activity by dinitrophenylation seen at saturating 2-keto-3-deoxy-6-phosphogluconate concentration is attributed to a decrease in catalysis rather than to a decrease in binding of 2-keto-3-deoxy-6-phosphogluconate. The rate of tritium exchange from $T_2O$ into pyruvate decreased about 10-fold following dinitrophenylation. Since tritium exchange in native 2-keto-3-deoxy-6-phosphogluconate aldolase is only 2 to 3 times the rate of cleavage, the 10-fold loss of activity may be attributed to either direct or indirect impairment of the C–H bond cleavage part of the reaction mechanism.

Discrepancies between the rate and extent of dinitrophenylation and the loss in activity were observed. These are attributed to a conformational change elicited by addition of the first molecule of fluorodinitrobenzene. An altered conformation is also supported by the unexpected change in $p$H to a more basic region, increased thermal stability, and decreased number of sulfhydryl groups available for titration with dithiodinitrobenzoate.

The data do not support the hypothesis that dinitrophenylation impairs the substrate phosphate group binding site but, rather, that it effects changes in conformation which indirectly diminish catalysis.

Fluorodinitrobenzene has been a useful probe in establishing the presence and function of certain amino acids in the active site of enzymes. In ribonuclease, dinitrophenylation of the lysine-41 ε-amino group resulted in inactivation. The reaction with 1-fluoro-2,4-dinitrobenzene was inhibited by phosphate and sulfate ions; this led Hirs, Halmann, and Kycia (1) to postulate that the lysine residue is involved in binding of a phosphate group on the substrate.

Rowley, Tchola, and Horecker (2) inactivated fructose diphosphate aldolase and transaldolase with FDNB or 1-chloro-2,4-dinitrobenzene with the arylation reaction occurring at cysteine and lysine residues, respectively. The reaction with transaldolase was also inhibited by phosphate and sulfate ions. In the case of transaldolase also, the data were interpreted as a reaction with a lysine residue involved in binding the phosphate group of the substrate.

Similar experiments were performed with crystalline 2-keto-3-deoxy-6-phosphogluconate aldolase of Pseudomonas putida. Ingram and Wood (3) observed that the reaction of fluorodinitrobenzene with KDGP aldolase at pH 8.0 resulted in the uptake of 4 moles of FDNB per mole of enzyme; this was accompanied by complete inactivation. Protection against dinitrophenylation and inactivation was afforded by 0.02 μ M KDGP or inorganic phosphate (Pi), but not by pyruvate. The sites of dinitrophenylation were identified as the ε-amino group of lysine residues; however, the lysine residues involved in catalysis through azomethine formation did not appear to be involved. On the basis of the above results, it was suggested that FDNB may be reacting at sites responsible for binding the phosphate group of KDGP (3).

If the above hypothesis were true, it should be possible to introduce a $^{14}C$-dinitrophenyl marker at the active site. Subsequent degradation of the labeled enzyme and amino acid sequencing of the labeled peptide would give information concerning...

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1 The abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; KDGP, 2-keto-3-deoxy-5-phosphogluconate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); KDP-Gal, 2-keto-3-deoxy-6-phosphogalactonate; DNP-, 2,4-dinitrophenyl.
ing the amino acids that comprise a portion of the active site. This information would be especially valuable since the sequence of a hexadecapeptide containing the asomethionine lysine has already been established (4). The present study was undertaken to determine more definitively whether FDNB reacted at sites on the enzyme responsible for binding the substrate phosphate group.

**EXPERIMENTAL PROCEDURE**

Enzymatic—Crystalline KDPG aldolase was prepared from P. putida as described by Ingram and Wood (5) and assayed as described by Meloche and Wood (6). 2-Keto-3-deoxy-6-phosphogalactonate aldolase was purified from *Pseudomonas saccharophila* as described by Schuster (7). In order to remove contaminating KDPG aldolase from this preparation, the procedure was followed through the acid precipitation step and then the acid precipitation step was repeated four more times. Following this, the KDPG aldolase activity was 0.1% of the original value.

Analytical—The dinitrophenylation reaction was followed spectrophotometrically by measuring the optical density at 360 nm in a Gilford recording spectrophotometer. In the early part of the reaction, the cuvette positoner was set to cycle every 5 min and, later, every 10 min. Enzyme activity was monitored by removing samples from the reaction cuvette at suitable intervals and assaying for enzyme activity. A control containing all additions except aldolase was run at the same time to determine the extent of nonenzymatic hydrolysis of FDNB. The uptake of FDNB was calculated using a molar extinction coefficient of 17,000 (8). The reaction mixture contained 250 μmoles of imidazole buffer (pH 8.5), about 1.0 x 10^-3 moles of aldolase, and 25 x 10^-3 moles of FDNB in a total volume of 0.25 ml. The total increase in 360 nm absorption of the control was approximately one-sixth of that obtained when enzyme is present. When FDNB-14C was used, its uptake was estimated after completion of the reaction as follows: the labeled enzyme was precipitated with trichloroacetic acid, the precipitate washed six times with 5% trichloroacetic acid, finally dissolved in 0.1 N NaOH, and a suitable aliquot was monitored for radioactivity.

The reductive binding of pyruvate to KDPG aldolase with sodium borohydride was accomplished as described by Ingram and Wood (3).

The rate of incorporation of tritium from T2O into pyruvate was measured in the following manner. The incubation mixture at 28° contained the following: 20 μmoles of pyruvate; 3 x 10^-4 moles of aldolase; 100 μmoles of imidazole buffer, pH 8.0; and 20 mCi of T2O in a total volume of 1.0 ml. Aliquots were removed at suitable intervals and the pyruvate was immediately converted to lactate by lactic dehydrogenase and NADH. The resulting mixture was then lyophilized, and the residue was dissolved in water. In order to remove all traces of T2O, the residue was alternately lyophilized and redissolved in water six times.

Isoelectric focusing was carried out in a 110-ml column at 2° for 40 to 48 hours. The ampholine gradient was prepared as described by the instruction bulletin for the LKB 8101 electrofocusing column, LKB Instruments, Rockville, Maryland.

The running gel for disc gel electrophoresis was prepared as described by Davis (9); no sample or spacer gel was employed. The enzyme (in 5% sucrose) was layered on top of the gel column. Tris-glycine buffer, pH 8.0, was used for disc gel electrophoresis and was prepared as described by Davis (9). All gels were polymerized in quarta tubes with a square cross-section and were subjected to a 30-min preliminary electrophoresis period to remove ultraviolet-absorbing impurities. After sample application, the gels were subjected to electrophoresis for 30 to 60 min using a current of 5 ma per tube. The gels were then directly scanned in a Gilford recording spectrophotometer equipped with a gel scanner attachment.

Heat inactivation of KDPG aldolase was effected by placing the enzyme (1 x 10^-4 μmoles) in 0.005 M phosphate buffer, pH 6.0 (preheated to 70°), to give a final volume of 1.0 ml. The test tube was then maintained at 70° in a water bath.

Sulhydryl group determinations were performed with 3,5-dithiobisnitrobenzoic acid as follows. The reaction mixture contained 0.04 ml of 1 mM DTNB in 5 mM phosphate buffer, pH 7.0; 0.14 ml of 0.5 M phosphate buffer, pH 8.0; about 100 μg of KDPG aldolase in a total volume of 0.3 ml. The control contained all the above additions except enzyme. Uptake of DTNB was calculated using a molar extinction coefficient of 13,500 for the DTNB anion (10). This value was verified using cysteine as a standard.

Radioisotope measurements were performed in a Packard Tri-Carb scintillation counter using the solvent system of Bray (11). Internal standards were included in order to correct for quenching of radioactivity.

Chemical—KDPG was prepared according to the procedure of Meloche and Wood (12). KDP-Gal was synthesized in a similar manner by incubating pyruvate and glyceraldehyde-3-P with KDP-Gal aldolase from *P. saccharophila*.

To facilitate more efficient incorporation of pyruvate-3-14C into KDP-Gal, a molar ratio of dl-glyceraldehyde-3-P to pyruvate of 3 was used. Following incubation with KDP-Gal aldolase, the product was precipitated twice with barium acetate and ethanol. The preparation was dissolved and then incubated with KDPG aldolase to destroy any traces of KDPG present. The resulting mixture was twice precipitated with the barium salt by addition of ethanol, and then chromatographed on Dowex 1-chloride with a linear gradient of 0 to 0.2 N HCl. The tubes containing KDP-Gal, as determined by reaction with semicarbazide (13) and activity with KDP-Gal aldolase (7), were pooled and the phosphate ester obtained as the barium salt. The preparation was 70% pure without correction for moisture and was devoid of pyruvate and KDP-Gal aldolase as determined in a spectrophotometric assay with NADH lactic dehydrogenase and KDPG aldolase.

The authenticity of the sample was determined by showing its ability to serve as a substrate for KDP-Gal aldolase but not KDPG aldolase. The dephosphorylated product (2-keto-3-deoxygalactonate) was thioarbituric acid-positive and released α-formylpyruvate at a much slower rate compared with 2-keto-3-deoxygluconate when treated with periodate (14). In addition, degradation of 2-keto-3-deoxygalactonate by borohydride reduction followed by a Ruff degradation (15) resulted in a compound that cochromatographed with authentic 2-deoxyxyllose. The specific activity of KDP-Gal-3-14C prepared in this manner using pyruvate-3-14C was 2.81 x 10⁶ dpm per pmole.

2-Keto-3-deoxygluconate and 2-keto-3-deoxygalactonate were obtained by treating the respective phosphorylated substrates...
with alkaline phosphatase followed by filtration on Sephadex G-25. A sample of authentic KDPG-Gal was generously donated by Dr. M. Doudoroff of the University of California at Berkeley, and Dr. J. Preiss of the University of California at Davis kindly donated a sample of 2-deoxyxyllose. All other chemicals were obtained from commercial sources.

**RESULTS**

**Conditions for Dinitrophenylation—**All attempts to repeat the dinitrophenylation of KDPG aldolase under the conditions described by Ingram and Wood (3) at pH 8.0 (in imidazole buffer), or at pH 9.1 (in bicarbonate buffer) led to the uptake of FDNB in excess of 4 moles of FDNB per mole of enzyme. The left side of Fig. 1 shows an example of dinitrophenylation at pH 9.1 and at room temperature. The reaction mixture contained 200 μmoles of bicarbonate buffer, 1.6 × 10^−3 μmoles of dialyzed KDPG aldolase, and 0.5 μmole of FDNB in a total volume of 1.0 ml. Samples were withdrawn at regular intervals for determination of KDPG aldolase activity. A control was run under identical conditions except that enzyme was omitted. ○, fluorodinitrobenzene consumed; ●, activity. The right-hand portion shows the reaction at pH 8.5 and 33°. The reaction mixture contained 250 μmoles of imidazole buffer, pH 8.5, about 1.0 × 10^−3 μmoles of aldolase, and 2.5 × 10^−4 μmoles of FDNB in a total volume of 0.25 ml. ○, absorbance; ●, activity; △ and ▲, effect of adding either KDPG or P, at 0.02 μm concentration on absorbance and activity, respectively. The right and left panels of the dual scale for the ordinate at the left apply to the right and left panels of the figure, respectively.

**Fig. 1.** Reaction of KDPG aldolase with FDNB. The left-hand portion shows the reaction at pH 9.1 and at room temperature. The reaction mixture contained 200 μmoles of bicarbonate buffer, 1.6 × 10^−3 μmoles of dialyzed KDPG aldolase, and 0.5 μmole of FDNB in a total volume of 1.0 ml. Samples were withdrawn at regular intervals for determination of KDPG aldolase activity. A control was run under identical conditions except that enzyme was omitted. ○, fluorodinitrobenzene consumed; ●, activity. The right-hand portion shows the reaction at pH 8.5 and 33°. The reaction mixture contained 250 μmoles of imidazole buffer, pH 8.5, about 1.0 × 10^−3 μmoles of aldolase, and 2.5 × 10^−4 μmoles of FDNB in a total volume of 0.25 ml. ○, absorbance; ●, activity; △ and ▲, effect of adding either KDPG or P, at 0.02 μm concentration on absorbance and activity, respectively. The right and left sides of the dual scale for the ordinate at the left apply to the right and left panels of the figure, respectively.

Following a reinvestigation of the conditions of dinitrophenylation, the experimental procedure was modified with the result that the aldolase reacted with a limited number of dinitrophenyl groups, as previously reported. Using the reaction conditions described under “Experimental Procedure” and based upon an average of 10 determinations, 3.8 ± 0.1 dinitrophenyl groups were introduced, with an 85 to 90% loss of activity. The range of these determinations was 3.7 to 3.9 moles of FDNB per mole of aldolase. The right side of Fig. 1 shows the course of dinitrophenylation was essentially complete within 60 min; at this point, 85% of the enzyme activity was lost. In the presence of 0.02 μM KDPG, or inorganic phosphate, dinitrophenylation of KDPG aldolase was almost completely inhibited and activity was correspondingly preserved as has been previously reported (3). Although not shown in Fig. 1, it was also found that 0.02 μM KDP-Gal inhibited dinitrophenylation and preserved activity in exactly the same manner.

In two similar experiments using 3H-FDNB and the same incubation conditions except that a 50-fold larger scale, it was found that 3.5 moles of 3H-FDNB per mole of aldolase were incorporated, and 89 and 88% of the activity was lost, respectively. These values agree well with those determined spectrophotometrically.

**Specificity of Protection Against Dinitrophenylation**—The previously observed protection against dinitrophenylation by KDPG and P, (3) was interpreted to indicate that dinitrophenylation was occurring almost exclusively at the active site, probably at the substrate phosphate binding site of the enzyme. It has now been found, however, that 0.02 μM glucose 1-phosphate, which is only a very weak competitive inhibitor ($K_i = 1 \times 10^{-2}$ μM) of KDPG aldolase activity, affords substantial protection against dinitrophenylation at concentrations much below the $K_i$ value. Specifically, with 0.02 μM glucose 1-P, 2 moles of FDNB were incorporated per mole of aldolase compared with 3.8 moles per mole for the native enzyme. This finding raised the question as to whether dinitrophenylation occurs outside of the active site, in which case the observed protection by relatively high concentrations of phosphate compounds (such as glucose-1-P, P, KDPG, or KDP-Gal) could result from a nonspecific ionic binding and direct protection in regions unrelated to the active site. In such a case, the loss of activity on dinitrophenylation would indirectly result from conformational changes. Alternatively, the lysine residue dinitrophenylated may be in the active site where arylation would result in activity loss directly. In this case, protection by phosphate compounds binding outside the site would result indirectly from a conformational change, thereby masking the potentially available lysines (see “Discussion”).

A test of these possibilities involved the use of the substrate analogue, 2-keto-3-deoxy-6-phosphogluconate, which is a competitive inhibitor ($K_i = 1 \times 10^{-2}$ μM) of KDPG aldolase activity which completely inhibits dinitrophenylation. Further, it had been established that KDP-Gal was not cleaved by the aldolase. If it could be shown that KDP-Gal forms a Schiff base with the aldolase, then reductive binding of KDP-Gal to the aldolase with NaBH₄, followed by dialysis, should yield an aldolase preparation with the active site occupied by covalently bound KDP-Gal, but with all other potential dinitrophenylation sites available for reaction. Treatment with FDNB should then show the amount of nonspecific arylation.

Table I reports the results of KDP-Gal binding to KDPG aldolase in the presence of sodium borohydride. In two separate experiments, KDPG aldolase bound 2.63 and 2.72 moles of KDP-Gal per mole of enzyme with complete loss in activity. In a separate control experiment, the radioactivity trapped in the absence of NaBH₄ was negligible (about 0.2% of the test with NaBH₄); also, it was shown that following treatment of the enzyme with unlabeled KDP-Gal and NaBH₄, pyruvate-3-¹⁴C does not bind in the presence of sodium borohydride. This finding indicates that KDP-Gal and pyruvate compete for a common azomethine site on the enzyme. The binding of about 3 moles of KDP-Gal to the aldolase is consistent with the data obtained on pyruvate reduction with NaBH₄ and is in agreement with the recent finding that KDPG aldolase is composed of three identical, or nearly identical, subunits (16, 17). The complete loss of activity on KDP-Gal binding as well as the stoichiometry of
These results show that occupation of the catalytic sites with per mole of enzyme, while the native enzyme bound 3.6 to 3.7 moles of FDNB per mole of enzyme under identical conditions. In both experiments, the treated KDPG aldolase contained 3.24 and 2.78 moles of KDP-Gal per mole of enzyme, respectively. In two separate experiments shown in Table II, the coupled enzyme was then incubated with FDNB in the usual changes of 0.05 M imidazole buffer, pH 8.0. The dinitrophenylation and analyses were performed as described under "Experimental Procedure." Calculations are based on a specific activity of KDPG-3-W of 2.81 X 10^5 dpm/mole.) was treated with KDP-Gal and NaBH₄ and finally followed by a single NaBH₄ addition. The control was treated in an identical manner except that NaBH₄ was not added.

### Table I

| Inactivation | KDP-Gal-3-¹⁴C bound |
|--------------|---------------------|
| %            | moles/mole aldolase |
| Experiment 1 | 100                 | 2.63 |
| Experiment 2 | 100                 | 2.72 |
| Control      | 3                   | 0.004 |

### Table II

| Dinitrophenylation of KDPG aldolase derivatized with KDP-Gal | Reduction binding | Dinitrophenylation |
|-------------------------------------------------------------|------------------|--------------------|
| Additions (KDP-Gal-3-¹⁴C bound)                             | No addition      | 0.02 M KDPG        |
| Experiment 1 NaBH₄                                         | 3.24             | 0.82               | 0.18 |
| None                                                       | 0.00             | 3.66               | 0.39 |
| Experiment 2 NaBH₄                                        | 2.78             | 0.75               | 0.52 |
| None                                                       | 0.00             | 3.88               | 0.58 |

*For Experiment 2, 1.06 mg of KDPG aldolase (1.599 X 10^2 μmoles) was treated with KDP-Gal and NaBH₄ and finally taken up in 1.0 ml of H₂O. A 0.04-m1 aliquot (6.3 X 10^2 μmoles of aldolase) bound 3089 cpm. After correcting for efficiency and quench, this value became 4695 cpm. From the specific activity of the ³⁴C-KDPG-aldolase, this corresponded to 16.71 X 10^4 μmoles or 2.78 moles of KDP-Gal bound per mole of aldolase.

KDP-Gal binding demonstrates that all three catalytic sites can be blocked by KDP-Gal.

Following dialysis over a 24-hour period against several changes of 0.05 M imidazole buffer, pH 8.5, the KDP-Gal-3-¹⁴C-coupled enzyme was then incubated with FDNB in the usual way. In two separate experiments shown in Table II, the treated KDPG aldolase contained 3.24 and 2.78 moles of KDP-Gal per mole of enzyme, respectively. In both experiments, the KDP-Gal-coupled enzyme bound less than 1.0 mole of FDNB per mole of enzyme, while the native enzyme bound 5.0 to 3.7 moles of FDNB per mole of enzyme under identical conditions. These results show that occupation of the catalytic sites with KDP-Gal abolishes the uptake of about 3 molecules of FDNB. The single molecule of FDNB bound by KDP-Gal-coupled enzyme probably represents dinitrophenylation outside of the active site and possibly reaction in a very small number of unoccupied active sites. It is noteworthy that reaction of the single molecule of FDNB with KDP-Gal-coupled enzyme is abolished by 0.02 M KDPG.

**Identification of Sites of Dinitrophenylation**—Identification of the dinitrophenyl amino acids involved acid hydrolysis of μC labeled dinitrophenyl aldolase. On acidification of the hydrolyzed enzyme and subsequent ether extraction, all of the radioactivity was found in the aqueous layer. Paper chromatography of an aliquot using butanol-acetic acid-water (4:1:5, lower phase or upper phase) and ethyl acetate-acetic acid-water (13:1:3, upper phase) showed the presence of a single radioactive spot with an Rf value in each solvent system identical with that of authentic ε-amino DNP-lysine and markedly different from the DNP-derivatives of cysteine and tyrosine, arginine and histidine.

Treatment of DNP-protein with mercaptoethanol has been shown to convert any DNP-cysteine present to cysteine (18). Incubation of the dialyzed, dinitrophenylated aldolase with mercaptoethanol over a 3-hour incubation period, as described by Shaltiel (18) did not increase enzyme activity. In addition, dialysis of the mercaptoethanol-treated enzyme did not result in the loss of yellow color from the DNP-aldolase. These results constitute additional evidence that the sulfhydryl groups of cysteine were not sites of dinitrophenylation. Aldolase containing 2 to 3 molecules of pyruvate reductively bound with NaBH₄ was still capable of binding approximately 4 moles of FDNB per mole of enzyme. Hence, the lysine residues dinitrophenylated were not those involved in azomethine formation. These data on the identification of certain lysine residues as sites of dinitrophenylation are in agreement with earlier results (3) derived from dinitrophenylation under somewhat different conditions.

**Physical State of Dinitrophenyl Aldolase**—The effect of dinitrophenylation on the physical state of the enzyme was examined by polyacrylamide gel electrophoresis and sucrose density gradient centrifugation. The scan at 280 mμ of the gel containing native enzyme subjected to 30-min electrophoresis at pH 8.0 showed a single protein peak. DNP-aldolase showed a single protein peak of the same Rf as native enzyme when scanned at both 280 mμ and 360 mμ. Native and DNP-aldolases had identical S values (4.2 S) on sucrose gradient centrifugation. These data indicate that DNP-aldolase has not undergone any gross changes in physical structure.

**Kinetics of Dinitrophenylation**—Murdock, Grist, and Hirs (19) and Levy and Li (20) have reported that the reaction between FDNB and ε-amino groups of lysine normally shows second order kinetics. The data presented in Fig. 2 was analyzed by the following equation:

\[
\log \frac{(A)}{(B)} = kt \left[ \frac{(B)}{(A)} \right] - \log \frac{(A)}{(B)}
\]

where A and B are the respective concentrations of the two reactants at any time, \( t \), during the reaction; \( (A) \) and \( (B) \) are the concentrations of the reactants at time zero; \( k \) is the second order rate constant and has units of \( \text{M}^{-1} \text{min}^{-1} \). The kinetic data for dinitrophenylation of the reactive lysines of KDPG aldolase were plotted in Fig. 2 where \( (A) \) is the concentration of lysine residues at time zero (4 lysines were assumed to react per molecule of enzyme); \( (B) \) is the concentration of FDNB at time zero; \( A \) and \( B \) are the respective concentrations of the reactants at any time, \( t \).
subtracting the values of \((A)/(B)\) along OM from those at reaction of the single fast reacting lysine residue is obtained by lysine can now be computed. Since LM represents the reaction of the slower reacting lysines (MN) to be 0.16 (L, Fig. 2). Extrapolation of that portion of the line representing the reaction of the slower reacting lysines (OM) to time zero (OM) intersects the ordinate at a point corresponding to a value for \((A)/(B)\) of 0.12. The resultant line ON represents the slower reacting lysines. Since 0.12 is 75\% of 0.16 (the initial \((A_1)/(B_1)\) ratio), this indicates that 3 of the 4 reactive lysines are represented by the line \(OM\) and by difference that there is a single fast reacting lysine. The kinetic data for the fast reacting lysine can now be computed. Since LM represents the reaction of the single fast reacting lysine as well as the reaction of the slower reacting lysines, and \(OM\) represents the reaction of the three slower reacting lysines over the same time period, the reaction of the single fast reacting lysine residue is obtained by subtracting the values of \((A)/(B)\) along \(OM\) from those at similar time intervals along \(LM\). The resulting values for the fast reacting lysine were then plotted in Fig. 2 (\(\triangle\)). The second

\[
\text{order rate constant for the fast reacting lysine was found to be 0.078 m}^{-1}\text{ min}^{-1}, \text{ while the second order rate constant for the slower reacting lysines was calculated to be 0.015 m}^{-1}\text{ min}^{-1}. \text{ Thus, the single fast reacting lysine residue is about 5 times more reactive than the three slower reacting lysine residues.}
\]

The kinetic data for the loss of enzyme activity is also plotted in Fig. 2 (\(\bullet\)). From the plot (PQR), it is evident that the reaction is biphasic, with an initial fast rate of inactivation which appreciably decreases after the first 10 min of reaction. The analysis of the data to determine the rate of the initial rapid inactivation is similar to the procedure used to determine the rate of reaction of the fast reacting lysine. Extrapolation of the line QR to the ordinate (S) gives the reaction of the slower loss of activity from time zero. The value of \((A)/(B)\) at \(S\) is 0.02 which is 50\% of the initial value of 0.04. This indicates that 50\% of the enzyme activity is rapidly lost on dinitrophenylation while subsequent enzyme inactivation proceeds at a slower rate. Since PO represents both the rapid and slow rates of inactivation and SQ gives the contribution of the slow loss of enzyme activity over the same time period, subtraction of the values of \((A)/(B)\) along \(SQ\) from the values along \(PQ\) at the same time intervals gives the course of the rapid enzyme inactivation. The resultant data is plotted in Fig. 2 (\(\square\)). It is immediately obvious that the rate of dinitrophenylation of the fast reacting lysine residue parallels the rapid rate of loss of 50\% of the enzyme activity. The data, therefore, indicate that the dinitrophenylation of a single reactive lysine residue results in 50\% inactivation of the enzyme activity.

It has been established that KDPG aldolase is composed of three identical, or nearly identical, subunits containing three substrate binding sites, and, presumably, three catalytic sites (16, 17). It would be expected, therefore, that if a single critical residue were destroyed on any subunit, the extent of inactivation should not exceed 35\% (one of three active sites destroyed). Thus, the observation that the uptake of a single mole of FDNB results in 50\% inactivation of the enzyme leads to the conclusion that the introduction of a single DNP group causes a conformational change leading to decreased enzyme activity in at least one of the two remaining catalytic sites.

**Nature of Residual Activity of Dinitrophenylated Aldolase**—It was repeatedly observed that treatment with FDNB abolished all but about 10 to 15\% of the activity. In order to assess the role of the lysine residues arylated in catalysis, it was necessary to determine if the residual enzyme activity represented native enzyme, partially dinitrophenylated enzyme, or a combination of these species. To resolve this question, the dinitrophenylated aldolase was examined by isoelectric focusing. As shown in the upper part of Fig. 3, native aldolase focused as a single peak with a pI (isoelectric point) of 4.8, whether determined as absorbance at 280 nm or as activity. Of the enzyme activity placed on the column, 98\% was recovered.

Under identical conditions, the DNP-aldolase, which is relatively insoluble, precipitated during the run and fell to the bottom of the column. However, with 4\% ampholine, the DNP-aldolase remained in solution. The lower part of Fig. 3 shows that DNP-aldolase was resolved into two separate peaks. The two pooled peaks were dialyzed prior to further analysis. Table I summarizes the properties of the two enzyme fractions. The minor, more acidic peak was located in the region occupied by native aldolase and consisted of about 5\% of the total enzyme placed on the column. This enzyme focused at about pH 4.8, had a
specific activity of 3800, and contained 1.6 moles of DNP-group per mole of enzyme. This minor peak may contain a small amount of native enzyme and a number of partially dinitrophenylated species. The major protein peak focused at pH 5.2 and contained 3.06 moles of FDNB per mole of enzyme. This protein peak comprised 95% of material placed on the gradient and had a specific activity of 800; the specific activity of native enzyme is 13,000. These results show that the fully dinitrophenylated enzyme, containing 4 moles of FDNB per mole of enzyme, still retains 6 to 7% of its initial activity. The data also show that the peaks were clearly resolved, thus minimizing the possibility that the major peak may be contaminated with native enzyme.

It should be noted that the higher degree of dinitrophenylation causes an increase in pI. Any lysine residue susceptible to FDNB must either be uncharged or have only a moderate degree of positive charge, whereas dinitrophenylated lysine can only be uncharged. Thus, the DNP-enzyme would be expected to focus either at the same pH as native enzyme, or at a lower pH. This apparent inconsistency of moving to a region of higher pH can be explained by assuming that the enzyme had undergone a conformational change causing an apparent change in pI. This assumption is in accord with the data obtained from the kinetic analysis of the dinitrophenylation reaction which indicated that the enzyme had undergone a change in conformation during dinitrophenylation. Attempts to substantiate this conclusion will be discussed below.

All subsequent experiments with DNP-aldolase were carried out with the major fraction from the electrofocusing column.

**Effect of Dinitrophenylation on $K_m$ and $V_{max}$**—The $K_m$ values of native and DNP-aldolase for KDPG were compared in order to determine whether the loss of activity on dinitrophenylation was due to the decreased ability of the enzyme to bind substrate ($K_m$), or to a decrease in catalytic activity ($V_{max}$). From two determinations each, it was found that the $K_m$ values for native aldolase were 0.72 and 0.74 x $10^{-4}$ M, while those of DNP-aldolase were 1.16 and 1.24 x $10^{-4}$ M. The almost 2-fold decrease in binding capacity for KDPG could not account for the loss of enzyme activity since the assays were performed at 5 to 10 times the $K_m$ level of substrate.

In view of the fact that DNP-aldolase has about the same $K_m$ for KDPG as native aldolase, it is concluded that dinitrophenylation must impair some phase of the catalytic process rather than the binding of substrate.

**Effect of Dinitrophenylation on Rate of Tritium Exchange**—Rutter, Richards, and Woodfin (22) have shown that the decreased activity of carboxypeptidase-treated fructose diphosphate aldolase was due to a drastic decrease in the rate of proton exchange into dihydroxyacetone phosphate to the point where this process limited the rate of cleavage. Since KDPG aldolase catalyzes an exchange of tritium into pyruvate (8), the loss of activity of KDPG aldolase upon dinitrophenylation could also result from a similar impairment. To test this possibility, the rates of incorporation of tritium from $T_2O$ into pyruvate for native and DNP aldolase were compared as an indication of a similar impairment of KDPG cleavage. The incubation mixture contained 20 pmoles of pyruvate, about 3 x $10^4$ pmoles of enzyme, 100 pmoles of imidazole buffer (pH 8.0), 20 mCi of $T_2O$ in a total volume of 1.0 ml. Aliquots were removed at regular intervals, and the pyruvate was converted to lactate by lactic dehydrogenase and NADH. The mixture was then lyophilized and the resulting residue redissolved. This procedure was repeated a total of six times to remove all traces of $T_2O$. Lactate determinations were made on the final solution by the procedure of Barker (23), and aliquots were assayed for radioactivity directly. The rate of incorporation of tritium into pyruvate by native enzyme was found in two determinations to be 0.20 to 0.29 $\mu$atom of tritium per min per i.u. of enzyme. These results are lower than, but consistent with, the rate of 0.41 $\mu$atom of tritium per min per i.u. of enzyme calculated from data published by Rose and O'Connell (24). From these values and an estimated isotope discrimination factor of 4.7 (24), the rate of tritium exchange into pyruvate was calculated to be 1.17 to 1.9 times the rate of cleavage of the substrate, KDPG. DNP-aldolase showed an exchange rate into pyruvate of 0.024 to 0.035

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**Table III**

Properties of dinitrophenyl-aldolases separated by isoelectric focusing

| Minor peak | Major peak |
|------------|------------|
| pH         | 4.8        | 5.2        |
| Amount of enzyme recovered (mg) | 0.16 | 2.9 |
| Specific activity | 3800 | 800 |
| FDNB content (moles per mole)* | 1.6 | 3.96 |

* Based upon amount of $^{14}C$-FDNB.
μatom tritium per min per i.u. of enzyme. This value is approximately 10% of the rate of exchange obtained with native enzyme. Moreover, DNP-aldolase cleaves the substrate KDPG at 6% of the rate of cleavage by native enzyme. Thus, the decrease in exchange and cleavage rates due to dinitrophenylation are roughly parallel.

**Conformational Changes**—The kinetics of dinitrophenylation and the anomalous pI of the DNP-aldolase indicated that the enzyme undergoes a conformational change on treatment with FDNB. Therefore, attempts to demonstrate this by observing changes in the rate of thermal inactivation and reaction with DTNB were undertaken.

Thermal inactivation was effected by incubating the enzyme in a 70°C water bath. The plots of the log of activity remaining versus time were linear for both native and DNP-aldolases, at least as far as 70 to 75% inactivation. The rate of inactivation of native enzyme was calculated to be 0.074 min⁻¹ versus 0.061 min⁻¹ for DNP-aldolase. These data show that the DNP-aldolase is slightly more heat stable than native aldolase.

Unpublished experiments from this laboratory have shown that native KDPG aldolase reacts with 5 moles of DTNB, while aldolase denatured in 8 M urea has an additional six to seven sulfhydryl groups available for reaction. Hence, the number of readily available sulfhydryl groups was used as a measure of conformational change. The reaction mixture contained 0.04 ml of 1 M DTNB in 5 M potassium phosphate, pH 7.0; 0.14 ml of 0.5 M potassium phosphate, pH 8.0; and 0.10 ml of KDPG aldolase containing 2 × 10⁻⁴ moles of enzyme in 0.005 M potassium phosphate buffer, pH 6.0, in a total volume of 0.3 ml. A control was also run containing all the above additions except enzyme. Under these conditions, native enzyme was found to react with 3.5 to 3.7 moles of Ellman's reagent, whereas the DNP-aldolase bound approximately 0.9 to 1.2 mole of DTNB per mole of enzyme.

**DISCUSSION**

Although it has been assumed that substrate protection against fluorodinitrobenzene is evidence of reaction in the active site, few experiments have been performed to eliminate other possibilities. With the observations that glucose l-phosphate (which binds outside the active site) and KDP-Gal (which binds in the active site) both protect against dinitrophenylation, there seems to be little possibility for a simple hypothesis which will accommodate the observations. When KDP-Gal was reductively bound and the excess removed, less than 1 equivalent of FDNB reacted. It is concluded that this is an extraneous reaction outside the active site and will not be considered further. The location of the three KDP-Gal-protectable residues must now be considered.

As shown in Table IV, three alternatives should be considered which depend upon whether the lysine residues dinitrophenylated are in the active site or outside the site. In each of the possibilities, a conformational change or other indirect effect must be postulated in order to accommodate the facts. The different models in Table IV derive from a consideration of which ligand, fluorodinitrobenzene, nonspecific phosphate compounds, or substrates (represented by KDP-Gal) cause the conformational change.

In the first model, it is postulated that the lysine residues dinitrophenylated are located outside the active site. Their protection results from nonspecific blocking of the lysine residue e-amino group in a direct sense by anions, most notably Pi and phosphate esters. The loss of activity on arylation would then be the indirect result of alteration of the active site; for instance, by conformational change. The experiments described herein seem to eliminate this possibility because occupancy of the active site by KDP-Gal under conditions which leave the presumed lysine residues outside the site available for dinitrophenylation resulted in dinitrophenylation of, at most, 1 lysine residue per mole. In the first model, all 4 lysine residues should have been available following reductive binding of KDP-Gal. It should be noted that oxidation or derivatization of —SH groups outside the active site by bisdithiodinitrobenzoate did not abolish activity and, hence, did not cause a conformational change of the type under consideration.

In the second model, the lysine residues are in the active site and their dinitrophenylation directly impairs activity. The protection by substrates and substrate analogues would be direct, whereas that of other phosphate compounds like glucose 1-phosphate would be indirect; for instance, via conformational change. In the latter case, this results from binding of these phosphate compounds outside of the active site. The evidence presented is consistent with this model in that KDP-Gal prevents the uptake of 3 molecules of FDNB, and this decrease is stoichiometrically equivalent to the number of sites.

The third model stipulates that the lysine residues are outside the site where binding of phosphate compounds accounts for the protection observed. The decrease in dinitrophenylation caused by reductive binding of KDP-Gal then indirectly results from a conformational change caused by the reductive binding of KDP-Gal in the site. The data reported herein do not eliminate this possibility; as a matter of fact, it has not been possible to eliminate this model for any enzyme where substrate protection of a reaction with an amino acid reagent has been observed. The essential difference between Models 1 and 3, as compared in this paper, is the fact that large numbers of glucose-1-P molecules can bind to produce the protection observed. With reductive binding of KDP-Gal, greater protection is observed with only 3

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4 K. A. Decker, H. Möhler, and W. A. Wood, unpublished experiments.
molecules of the substrate analogue bound per molecule of enzyme. For this reason, Model 2 appears to better fit the data than Model 3.

In this critical experiment, use was made of the unique property of KDP-Gal. Although not cleaved by KDPG aldolase, KDP-Gal forms an azomethine with the aldolase which can be fixed by treatment with NaBH₄. The reaction is specific in that about 3 molecules of analogue are bound and activity is completely lost in the process. KDP-Gal probably also binds non-azomethine-bound KDP-Gal and its reduction products can be removed by dialysis. Through the use of ¹⁴C-KDP-Gal, both the stoichiometry of covalent binding and the complete removal of all extraneous KDP-Gal can be ascertained. Following dialysis, all surface regions of the enzyme become available for dinitrophenylation except the active site occupied by KDP-Gal and fixed by NaBH₄ reduction.

If the stoichiometric and kinetic measurements are reasonably accurate, the kinetics of dinitrophenylation indicate that the reaction is not straightforward. This is shown by calculations involving extrapolation of rates in Fig. 2 to zero time which show that reaction of one fast reacting lysine residue results in a 50% loss in activity. In an aldolase with three identical or nearly identical subunits (16, 17), reaction of 1 lysine residue should result in 33% inactivation. Thus, it seems necessary to postulate cooperativity among subunits wherein a conformational change resulting from the first dinitrophenylation results in slower reactivity with FDNB in the subsequent reactions and also results in diminished activity in the two remaining subunits. It should be noted, however, that such cooperativity is not displayed as sigmoidal kinetics in enzyme assays.

Although it has been considered formerly that reaction with FDNB impaired binding of the substrate phosphate group to protonated e-amino groups of lysine residues (1 to 3), the data presented in this paper do not support such a hypothesis. If impairment of phosphate group binding occurred, a large increase in Kₘ for the substrate and a small change in Vₘₐₓ would have been expected. The opposite was actually observed. Thus, for KDPG aldolase, there is no longer evidence for a role of lysine residues in substrate phosphate group binding.

The 10-fold decrease in Vₘₐₓ indicates an important role of some nonazomethine-forming lysine residues in catalysis, very likely in that phase of catalysis which can be measured as tritium exchange into pyruvate. However, dinitrophenylation in the range reported here was incapable of complete abolition of activity. Following isoelectric focusing which removed native KDPG aldolase, the major peak, very likely a homogeneous species containing 4 lysine residues, still had more than 5% of the original activity. Thus, the cause of the decrease in Vₘₐₓ by FDNB may be indirect; that is, the result of structural change. Otherwise it would be expected that arylation of an amino group participating directly in the reaction should abolish activity completely and show a direct relationship between FDNB consumed and activity lost. As noted above, such a correspondence was not observed.

Although there was the possibility that the lysine e-amino group detected in this way could furnish the base needed either for proton exchange with pyruvate or for cleavage of KDPG, the data of Meloche (25) concerning the inactivation of KDPG aldolase with bromopyruvate can be interpreted as indicating that sulfhydryl and carboxyl groups may be properly located in the active site to serve these nucleophilic functions.

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The Mechanism of 2-Keto-3-deoxy-6-phosphogluconate Aldolase: III. NATURE OF THE INACTIVATION BY FLUORODINITROBENZENE
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