# Lysine-146 of Rabbit Muscle Aldolase Is Essential for Cleavage and Condensation of the C3–C4 Bond of Fructose 1,6-Bis(phosphate)†

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ABSTRACT: Lysine-146 of rabbit muscle aldolase (D-fructose-1,6-bisphosphate aldolase, EC 4.1.2.13) is absolutely conserved in class I (Schiff base) aldolases and has been implicated previously in catalysis by protein modification. Site-directed mutagenesis was used to change lysine-146 to alanine, glutamine, leucine, or histidine, creating the mutant enzymes K146A, K146Q, K146L, and K146H, respectively. These mutant proteins were expressed at high levels in bacteria and were purified by substrate affinity elution from CM-Sepharose, the same method that is used for the wild-type enzyme. The mutants K146A, K146Q, and K146L had substrate cleavage rates below standard detection levels. Modified cleavage assays indicated that these enzymes were (0.5–2) × $10^6$-fold decreased in the rate of catalysis of fructose 1,6-bis(phosphate) (Fru-1,6-$P_2$) cleavage. The K146H enzyme, however, was approximately 2000-fold slower than wild type in the rates of both cleavage and condensation of Fru-1,6-$P_2$. In assays for the presence of enzymatic intermediates, all of the mutant enzymes were able to catalyze formation of the carbanion intermediate with dihydroxyacetone phosphate, whereas this intermediate was below the level of detection with Fru-1,6-$P_2$. Single-turnover experiments with these enzymes in excess over radiolabeled Fru-1,6-$P_2$ were used to measure the rates of Schiff base and product formation. The rate of Schiff base formation was decreased in each of the mutant enzymes, yet the magnitude of this decrease was less than the reduction in the respective $k_{cat}$. These mutations had a much larger effect, however, on the rate of C3–C4 bond breaking, showing that Lys-146 is crucial at this step of the catalytic cycle.

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) catalyzes the reversible aldol cleavage of Fru-1,6-$P_2$[1] into two triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, in an ordered uni-bi mechanism. Aldolases have been divided into two classes on the basis of their mechanism (Rutter, 1964). Class I aldolases, which are found in most eukaryotes, catalyze the reversible reaction through a Schiff base intermediate between an active site lysine and the carbonyl carbon of the substrate. This covalent intermediate can be trapped by reduction with sodium borohydride or by acid precipitation (Horecker et al., 1972; Kuo & Rose, 1985). Class II aldolases, found in bacteria and fungi, catalyze the reaction using a metal cofactor. Chelation of this catalytic metal by EDTA leads to complete inactivation of the class II enzymes (Rutter, 1964).

Vertebrate aldolases exist in three isozyme forms, A, B, and C, that are tissue specific and have activities that suit their particular metabolic roles (Penhoet et al., 1966). The aldolase from rabbit muscle, isozyme A, is the most extensively characterized class I aldolase (Horecker et al., 1972; Creighton & Murthy, 1990). Studies of this enzyme using protein-modifying reagents have identified several residues potentially involved in the catalytic mechanism (Kobashi & Horecker, 1967; Hartman & Welch, 1974). Indeed, a mechanism based on these studies has been proposed (Lai et al., 1974) and is found in many textbooks (Rawn, 1989; Voet & Voet, 1990). Besides the active site lysine, Lys-229, either of two cysteine residues (Cys-72/Cys-338) and a histidine at position 361 have been proposed to be involved directly in catalysis. The role of Lys-229 has been clearly established as the active site lysine that forms a Schiff base with the ketose substrate (Lai & Oshima, 1971). However, the role suggested for these other residues is not consistent with current evidence: these residues are not in the active site, as indicated by the 2.7-Å structure of rabbit aldolase A (Sygusch et al., 1987); they are not conserved in class I aldolases (Rottmann et al., 1984); and site-directed mutagenesis of these residues had little effect on activity (Takahashi et al., 1989; Berthiaume et al., 1991).

Site-directed mutagenesis of Asp-33 has previously been shown to drastically reduce the activity of the enzyme (Morris & Tolan, 1993). It was proposed that this residue may be involved in catalyzing cleavage of Fru-1,6-$P_2$ perhaps by increasing the $pK_a$ of the critical base responsible for abstraction of the C4-hydroxyl proton. This chemical step, or the release of the triose phosphate products, has been proposed as the rate-determining step for aldolase A (Grazi & Trombetta, 1980; Rose & Warms, 1985; Rose et al., 1987). In this study, characterization of enzymes mutated at lysine-146 shows that this lysine is critical for the chemical step in catalysis that leads to C3–C4 bond cleavage.

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[1] Abbreviations: Fru-1,6-$P_2$, fructose 1,6-bis(phosphate); Fru-1-P, fructose 1-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Mops, 3-(*N*-morpholino)propanesulfonic acid; TEA, triethanolamine; Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; BSA, bovine serum albumin.

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## MATERIALS AND METHODS

*Materials.* Restriction endonucleases, T4 DNA ligase, exonuclease III, and DNA polymerase I were from New England Biolabs. DNA polymerase I (Klenow fragment), calf intestine alkaline phosphatase, and glycerol-3-phosphate dehydrogenase/triosephosphate isomerase were from Boehringer Mannheim. Deoxynucleoside triphosphates, CM-Sepharose, and CL-6B Fast Flow were from Pharmacia. [$\alpha$-$^{32}$P]Deoxynucleoside triphosphates were from Amersham Corp. [U-$^{14}$C]Fru-1,6-$P_2$ was from ICN. Scintillation fluid (Aquasol) was from DuPont. Nitrocellulose filters were from Sartorius. TLC plates were from Whatman. Oligonucleotides for construction, sequencing, and site-directed mutagenesis were synthesized on Millipore DNA synthesizers using phosphoramidite chemistry and the manufacturer's protocols. When necessary, oligonucleotides were purified by urea–PAGE. SDS low-$M_r$ standards were from Bio-Rad. DHAP was from Fluka. Fru-1,6-$P_2$, Fru-1-P, G-3-P, and other chemicals were from Sigma.

*Strains. Escherichia coli* strain TG1 (Sambrook et al., 1989) was used for M13 cloning and mutagenesis. DH5$\alpha$ (Sambrook et al., 1989) was used for protein expression.

*Site-Directed Mutagenesis of Expression Plasmids.* Site-directed mutagenesis was performed (Taylor et al., 1985) to change the Lys-146 (AAG) codon to an Ala, a Gln, a Leu, or a His codon using the oligodeoxyribonucleotide 5′-GACTTCGCCNNNTGGCGTTGC, where NNN, was GCG, CAG, CTG, or CAC, respectively. The Lys-229 to Ala mutation, as well as the screening, subcloning, and DNA sequence confirmation of all mutations, was as described previously (Morris & Tolan, 1993).

*Purification of Recombinant Aldolase.* Aldolase expression and purification were as described (Morris & Tolan, 1993) with the following modifications. The French press lysate of bacteria overexpressing rabbit aldolase A was centrifuged for 30 min at 30000*g* and for 60 min at 100000*g*. The mutant enzymes were eluted from CM-Sepharose CL-6B Fast Flow with MG buffer (50 mM Mops/glycine/KOH, pH 7.0, and 1 mM dithiothreitol) containing 2.5 mM Fru-1,6-$P_2$. SDS–PAGE (12.5% acrylamide) was as described by Laemmli (1970).

*Structural Analysis of Recombinant Proteins.* The CD spectra were determined using a protein concentration of 4.0 mg/mL (Baranowski & Niederland, 1949) in 1 mM Tris·HCl, pH 7.5, at 20 °C on an AVIV 60DS spectrometer using a 0.1 mm path length cuvette. CD spectra were taken from 180 to 260 nm with the reading averaged for 5 s at each nanometer increment.

*Fructose 1,6-Bis(phosphate) Cleavage Assay.* The substrate cleavage rate was determined by measuring the decrease in $A_{340}$/min in a coupled assay. Aldolase was diluted in 50 mM TEA·HCl, pH 7.4, and added to a cuvette containing 50 mM TEA·HCl, pH 7.4, 10 mM EDTA, 0.16 mM NADH, and 10 $\mu$g/mL glycerol-3-phosphate dehydrogenase/triosephosphate isomerase. Assays (1 mL) were performed in triplicate at 30 °C following addition of substrate. The cleavage rate for Fru-1,6-$P_2$ was measured over a substrate concentration range of 1.25–2000 $\mu$M, with 0.3 $\mu$g of wild-type enzyme or with 0.1–1.5 mg of mutant enzymes. Protein concentration was determined by absorbance using $E_{280}$ (0.1%) = 0.91 (Baranowski & Niederland, 1949).

*End Point Triose Phosphate Assay.* Enzyme was incubated with Fru-1,6-$P_2$ for as long as 16 h. A 520-$\mu$L reaction mixture contained $8.0 \times 10^{-7}$ mg of wild-type or 0.35 mg of mutant aldolase and 2 mM Fru-1,6-$P_2$ in 50 mM Tris·HCl, pH 7.2, 1 mM EDTA, and 0.1 mg/mL BSA. After the appropriate reaction time 500 $\mu$L of assay cocktail (100 mM Tris·HCl, pH 7.3, 20 mM EDTA, 0.32 mM NADH, and 20 $\mu$g/mL glycerol-3-phosphate dehydrogenase/triosephosphate isomerase) was added to the reaction. This mixture was then added to a cuvette, and the absorbance at 340 nm was determined. This absorbance was subtracted from a control that did not contain Fru-1,6-$P_2$.

*Fructose 1,6-Bis(phosphate) Synthesis Assay.* Fru-1,6-$P_2$ synthesis was measured by an end point assay (Dische & Devi, 1960). A 250-$\mu$L assay contained 5 mM each of DHAP and G-3-P in 200 mM Tris·HCl, pH 7.4, and 0.1 mg/mL BSA. G-3-P was prepared from glyceraldehyde 3-phosphate diethyl acetal di(cyclohexylammonium) salt as described by Berthiaume et al. (1991), and dihydroxyacetone phosphate dilithium salt monohydrate was dissolved in water to the appropriate concentration. The reaction was started by addition of the substrates and stopped with 3 mL of sulfuric acid. The color reactions were started by addition of 50 $\mu$L of 25% cysteine–HCl. After the mixture was incubated for 3 h at 20 °C, the difference between the absorbance at 412 and 380 nm was measured. The amount of Fru-1,6-$P_2$ synthesized was calculated from a standard curve generated with 5–100 nmol of Fru-1,6-$P_2$.

*pH Dependence of Catalysis.* The pH dependence of the forward steady-state reaction was determined using the coupled cleavage assay described above with the following modifications. Enzyme (0.5–400 $\mu$g) was diluted (900 $\mu$L) in MMTG (50 mM each of MES, MOPS, TAPS, and glycine) that was adjusted to the desired pH with 1 M KOH and kept at a constant ionic strength (0.24 M) by addition of KCl, and contained 0.19 mM NADH and 20 $\mu$g/mL glycerol-3-phosphate dehydrogenase/triosephosphate isomerase. The reaction (1 mL) was started by addition of 50 mM Fru-1,6-$P_2$.

*Carbanion Oxidation.* The carbanion present at C3 of the substrates can be oxidized by hexacyanoferrate(III) (Healy & Christen, 1973). The rate of hexacyanoferrate(III) reduction, which is proportional to the concentration of carbanion, was monitored by the decrease in absorbance at 420 nm. The 1-mL assay mixture contained 200 mM Tris·HCl, pH 7.4, 0.7 mM hexacyanoferrate(III), 0.1 mg/mL BSA, 0.03–1.5 mg of purified aldolase, and either 5 mM Fru-1,6-$P_2$ or 5 mM DHAP. The rate of decrease in absorbance was measured within the first 30 s at 25 °C.

*Single-Turnover Analysis.* Reactions were started by adding [U-$^{14}$C]Fru-1,6-$P_2$ (235 Ci/mol) to a final concentration of 2.5 $\mu$M in a 100-$\mu$L reaction mixture containing 17.5 $\mu$M mutant enzyme subunits in 10 mM Tris·HCl, pH 7.4. This solution was incubated at 21 °C for 2.0–3000 s and quenched with 100 $\mu$L of 4 N HCl. The quenched reaction mixture was centrifuged for 30 s, and the supernatant was removed, frozen at −70 °C, and lyophilized. The centrifuge tube containing the precipitated protein was rinsed with 200 $\mu$L of 2 N HCl and dried by vacuum centrifugation. The amount of radiolabeled Fru-1,6-$P_2$ incorporated into the precipitated enzyme was determined by adding scintillation fluid to the dried precipitate and counting in a Beckman LS 2800 scintillation counter. Counting efficiency was 85%. In a range of 1–3.5 N HCl, a concentration of 2 N was optimal for intermediate trapping. The reactions with wild-type enzyme were the same except that 70-$\mu$L reactions were performed at 4 °C in a KinTek Instruments quench-flow apparatus, such that reaction times could be varied from 3.0 to 400 ms.

Product formation was measured by thin layer chromatography (TLC) analysis of the HCl quench supernatant. The lyophilized supernatant was resuspended in water, applied (33%) to a silica thin-layer plate, and chromatographed using 1-butanol/glacial acetic acid/water (5:3:2). The spots were quantitated with a Molecular Dynamics PhosphorImager. Control experiments were performed to ensure that the acid was quenching the reaction, to check the stability of the substrates under the quench conditions, and to determine the background of the assay. Under the conditions of the assay 30–60% of the substrate was incorporated.

*Data Analysis.* Steady-state kinetic values were determined from double-reciprocal plots using a least squares method as described by Cleland (1990). The single-turnover kinetic values were determined by modeling the data according to the irreversible reaction scheme

$$E + \text{Fru-1,6-P}_2 \xrightarrow{k_1} E{=}\text{Fru-1,6-P}_2 \xrightarrow{k_2} \text{G-3-P} + \text{DHAP} + E$$

where E=Fru-1,6-$P_2$ is the Schiff base intermediate whose rate of formation is $k_1$. The rate constant $k_1$ represents the combination of several rates: the bimolecular binding, the unimolecular conversion of bound enzyme–substrate complex to the first covalent carbinolamine intermediate, the conversion of this carbinolamine to the Schiff base, and any dissociation reaction. The model assumes that the rates of association and dissociation are relatively fast compared to the formation of covalent intermediates in the pre-steady-state conditions. Furthermore, since the conversion of carbinolamine to Schiff base is thought to be rapid (Rose et al., 1987; Sayer et al., 1974), all covalent intermediates are referred to as Schiff base. However, the carbinolamine is stable in the acid precipitate (Kuo & Rose, 1985), and for some mutant enzymes it is possible that the carbinolamine is the major covalent intermediate measured in this assay. The rate of generation of the products G-3-P and DHAP is $k_2$. The rate constant $k_2$ represents only the carbon–carbon bond cleavage step. Data was fit by an iterative method to the following equations (Fersht, 1985):

$$y = \frac{Ak_1}{k_2 - k_1}(\exp^{-k_1 t} - \exp^{-k_2 t}) \quad (1)$$

$$y = A\left[1 + \frac{1}{k_1 - k_2}[(k_2 \exp^{-k_1 t}) - (k_1 \exp^{-k_2 t})]\right] \quad (2)$$

The concentration of Schiff base–enzyme intermediate versus time was fit to eq 1 to determine the value of $k_1$. The amount of triose phosphate products versus time was fit to eq 2 to obtain the value of $k_2$. In these equations $y$ represents the observed concentration of Fru-1,6-$P_2$ covalent intermediate at time $t$ (eq 1) or the observed concentration of reaction products at time $t$ (eq 2), and $A$ represents the maximum amplitude of the change in these concentrations expected for the reaction.

## RESULTS

*Purification and Structural Measurements.* The mutant proteins K146A, K146Q, K146L, and K146H were created by using site-directed mutagenesis to replace the Lys-146 codon with Ala, Gln, Leu, and His codons, respectively. These mutant proteins were expressed in soluble form in *E. coli* from the expression vector pPB1 (Beernink & Tolan, 1992) at a level of 300 mg/L of culture. The DNA sequence of each plasmid construction confirmed that only the desired mutation was present (data not shown). Purification of the recombinant mutant aldolases was accomplished by the same two-step procedure that is used for wild type: ammonium sulfate fractionation followed by substrate affinity elution from CM-Sepharose (Morris & Tolan, 1993). The enzyme preparations were greater than 95% homogeneous, and their apparent molecular weight was the same as wild type as determined by SDS–PAGE (data not shown). To ensure that the mutations did not greatly interfere with the structural integrity of the proteins, each mutant aldolase was analyzed by circular dichroism. The CD spectra of the mutant and wild-type aldolases were nearly identical (Figure 1), indicating that there were no major secondary structural perturbations caused by these amino acid substitutions in the active site.

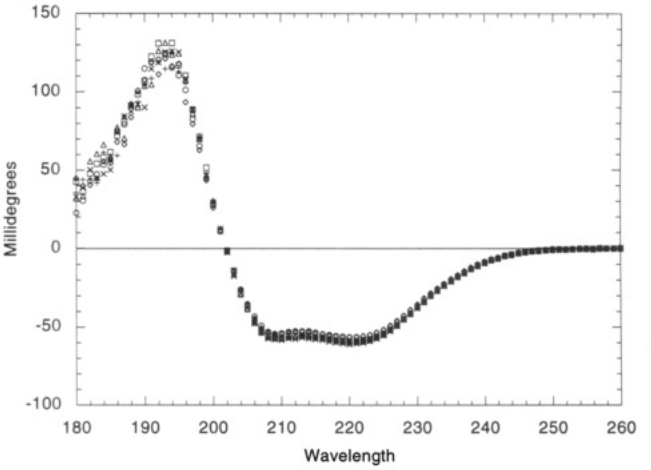


FIGURE 1: Circular dichroism spectra of wild-type and mutant aldolases at 20 °C. The spectra for wild type (○), K146A (×), K146Q (△), K146L (◇), K146H (□), and K229A (+) are shown.

Table 1: Steady-State Rates of Fructose 1,6-Bis(phosphate) Cleavage and Synthesis

| | cleavage | | | synthesis | |
|---|---|---|---|---|---|
| aldolase | $V_{max}$ (units/mg) | fold decrease | $K_m$ ($\mu$M) | sp act. (units/mg) | fold decrease |
| wild type | 20.8 ± 0.5 | | 14.3 ± 0.7 | 24.6 | |
| K146A | <0.0002 | >1 × $10^5$ | | *a* | >5 × $10^5$ |
| K146Q | <0.0002 | >1 × $10^5$ | | *a* | >5 × $10^5$ |
| K146L | <0.0002 | >1 × $10^5$ | | 0.0002 | >1 × $10^5$ |
| K146H | 0.0083 ± 4 × $10^{-4}$ | 2500 | 460 ± 30 | 0.011 | 2200 |
| K229A | <0.0002 | >1 × $10^5$ | | 0.0001 | >2 × $10^5$ |

[a] No Fru-1,6-$P_2$ detected after a 60-min reaction.

*Steady-State Kinetics.* The ability of the mutant aldolases to catalyze cleavage of Fru-1,6-$P_2$ was determined from a standard coupled assay that measures the triose phosphate products (Racker, 1952). The K146A, K146Q, and K146L enzymes had no detectable cleavage activity,[2] even with 1.5 mg of aldolase in the assay (Table 1). This result indicated that there was at least a $10^5$-fold decrease in $k_{cat}$ in the forward direction for these enzymes. Only the K146H enzyme retained any detectable activity in this assay. The $V_{max}$ of K146H was 2500-fold less, and the $K_m$ for Fru-1,6-$P_2$ was 30 times greater, than wild type. To determine whether K146A, K146Q, and K146L were inactive, a long-term end point assay was developed. After more than 13 h of incubation of enzyme

[2] The mutant protein K229A (Morris & Tolan, 1993) was used to define the background in all assays of turnover and enzymatic intermediates. This mutant has alanine substituted for the Schiff base forming lysine and therefore cannot catalyze the aldolase reaction via the normal mechanism.

Table 2: Cleavage Rates for Wild-Type and Mutant Enzymes in an End Point Coupled Assay

| aldolase | 13.2-h reaction (units/mg) | fold decrease | 15.8-h reaction[a] (units/mg) | fold decrease |
|---|---|---|---|---|
| wild type (pPB14) | 9.5 | | 7.7 | |
| K146A | $4.3 \times 10^{-6}$ | $2.2 \times 10^{6}$ | $3.6 \times 10^{-6}$ | $2.1 \times 10^{6}$ |
| K146Q | $1.0 \times 10^{-5}$ | $9.5 \times 10^{5}$ | $7.9 \times 10^{-6}$ | $9.7 \times 10^{5}$ |
| K146L | $1.4 \times 10^{-5}$ | $6.8 \times 10^{5}$ | $1.5 \times 10^{-5}$ | $5.1 \times 10^{5}$ |
| K229A | $2.7 \times 10^{-6}$ | $3.5 \times 10^{6}$ | nd[b] | nd |

[a] Incubated for 23 h without substrate prior to the 15.8-h reaction. [b] Not determined.

with substrate, small amounts of triose phosphate could be detected above background[2] for the mutant enzymes (Table 2). This assay indicated that these mutant enzymes had (5–20) $\times 10^{-7}$ the activity of wild type. The possibility that the extremely low rates reflected a loss of enzyme stability over such an extended reaction time was addressed by preincubating each enzyme under assay conditions for 23 h prior to initiating the reaction with substrate. Although the rates were slightly lower, the relative rates were very similar to those without preincubation (Table 2).

The rate of the reverse reaction, synthesis of Fru-1,6-$P_2$ from DHAP and G-3-P, was determined from an end point assay for Fru-1,6-$P_2$ (Table 1). The K146A, K146Q, and K146L mutants were at or below the detection level of the assay,[2] indicating that the reduction in catalysis caused by these substitutions was on the same order of magnitude in both directions. The rate of Fru-1,6-$P_2$ synthesis for K146H was 2200-fold decreased, which is similar to the extent of the activity deficit in the cleavage reaction. The severe decrease in turnover upon substitution of Ala, Gln, or Leu for Lys-146 indicated that Lys-146 was critical for catalysis in both directions of the aldolase reaction. The partial activity of K146H suggests that a positive charge at position 146 is important for catalysis, and indicates that replacement of Lys-146 does not simply inactivate the enzyme by perturbing the overall enzyme structure.

*Carbanion Intermediate Determination.* A more precise role of Lys-146 may be inferred by measurement of intermediates formed at different steps during the reaction (Scheme 1). In addition to the covalent Schiff base intermediate, an enamine/carbanion intermediate has been detected in the aldolase catalytic mechanism (Healy & Christen, 1973). The level of carbanion intermediate formed either from Fru-1,6-$P_2$ or DHAP can be measured from the rate at which it reduces hexacyanoferrate(III). Generation of a carbanion from Fru-1,6-$P_2$ requires formation of the Schiff base, abstraction of the C4-hydroxyl proton, and subsequent cleavage of the C3–C4 bond (top reaction, Scheme 1). Production of carbanion intermediate from DHAP is dependent upon Schiff base formation and deprotonation of C3 (bottom reaction, Scheme 1).

The rate of carbanion oxidation for the wild-type enzyme was nearly the same with either substrate; however, for the mutant enzymes the rate differed significantly with the two substrates (Table 3). None of the mutant enzymes had a significant level of carbanion intermediate with Fru-1,6-$P_2$.[2] Yet, when DHAP was the substrate, all of the mutant enzymes were able to form significant amounts of carbanion, although in all cases the rate of carbanion oxidation was 4–140-fold less than wild type. For K146A, K146Q, and K146L, the slower carbanion oxidation rates with DHAP cannot account for the negligible rate of Fru-1,6-$P_2$ synthesis. Furthermore, the fact that all of the Lys-146 mutants were able to form the carbanion intermediate from DHAP indicated that Lys-146 was not essential for formation of the DHAP Schiff base or for deprotonation of C3. The data suggests that the largest impact of substitution at Lys-146 is on steps leading to formation of the carbanion intermediate from Fru-1,6-$P_2$, which include Fru-1,6-$P_2$ Schiff base formation and C3–C4 bond cleavage.

*Single Turnover Analysis.* The $>10^6$-fold decrease in the forward $k_{cat}$ for the Lys-146 mutants was apparently due to a decrease in the rate of either formation of the Fru-1,6-$P_2$–Schiff base intermediate or cleavage of the C3–C4 bond (Scheme 1). The rates of both of these steps were measured using single-turnover experiments with enzyme in excess. These experiments monitored, as a function of time, incorporation of [U-$^{14}$C]Fru-1,6-$P_2$ into protein and generation of triose phosphate products prior to quenching with HCl. The amount of [U-$^{14}$C]Fru-1,6-$P_2$ incorporated into the protein increased to a maximal amount as the Schiff base intermediate was formed and subsequently decreased as cleavage occurred and G-3-P and DHAP were produced (Figure 2). When the aldolase reactions were quenched with HCl, the covalent intermediates that were present at the time of quenching remained attached to the precipitated protein (Rose & Warms, 1985; Rose et al., 1987), while the noncovalently bound substrates and products were released into solution. The wild-type rates were determined with a rapid-quench apparatus at 4 °C, while the pre-steady-state analysis of the mutants was performed at 21 °C on a time scale that did not require special equipment. Intermediates and products were monitored in the same reaction. This information provided some indication of what was being trapped on the enzyme, since the presence of product would indicate whether the radioactivity that precipitated with the enzyme was a six-carbon or a three carbon sugar. During the early phase of each time course, when the amount of covalent intermediate was increasing exponentially, there was no triose phosphate product present (Figure 2). This result indicated that it was the six-carbon intermediate that was being trapped and supported previous evidence that the rate of Fru-1,6-$P_2$–Schiff base formation was faster than the rate of product formation (Model et al., 1968).

Graphs of covalent intermediate and triose phosphate product concentration versus time were fit to equations for an irreversible two-step process and are shown in Figure 2. All of the mutants were able to form the covalent intermediate, except K229A (data not shown). The rate of Schiff base formation ($k_1$) was 95 $s^{-1}$ at 4 °C for the native enzyme and 0.004–0.17 $s^{-1}$ at 21 °C for the mutant enzymes (Table 4). These rates were 600–30 000-fold less than the rate determined for the wild-type protein, but could not account for the reduction in $k_{cat}$ for these mutants. Although substitution of Lys-146 had an effect on the rate of Fru-1,6-$P_2$–Schiff base formation, Lys-146 was not essential for formation of the critical Schiff base intermediate. In addition, enzymes with mutations at Lys-146 retain the ability to form a DHAP–Schiff base intermediate as demonstrated by the presence of carbanion from the DHAP substrate (Table 3).

The impact of the K146A, K146Q, and K146L mutations on product formation was immense. Although these mutants were able to form the Schiff base (or at least the carbinolamine as defined in Materials and Methods), cleavage of this intermediate to yield triose phosphate products (Scheme 1) was not detectable,[2] such that the data could not be fit to eq 2. The covalently trapped Schiff base for all three mutant enzymes reached a plateau in under 10 min, yet these enzymes were still unable to produce a significant amount of triose phosphate after an additional 40 min of reaction (Figure 2,

Scheme 1

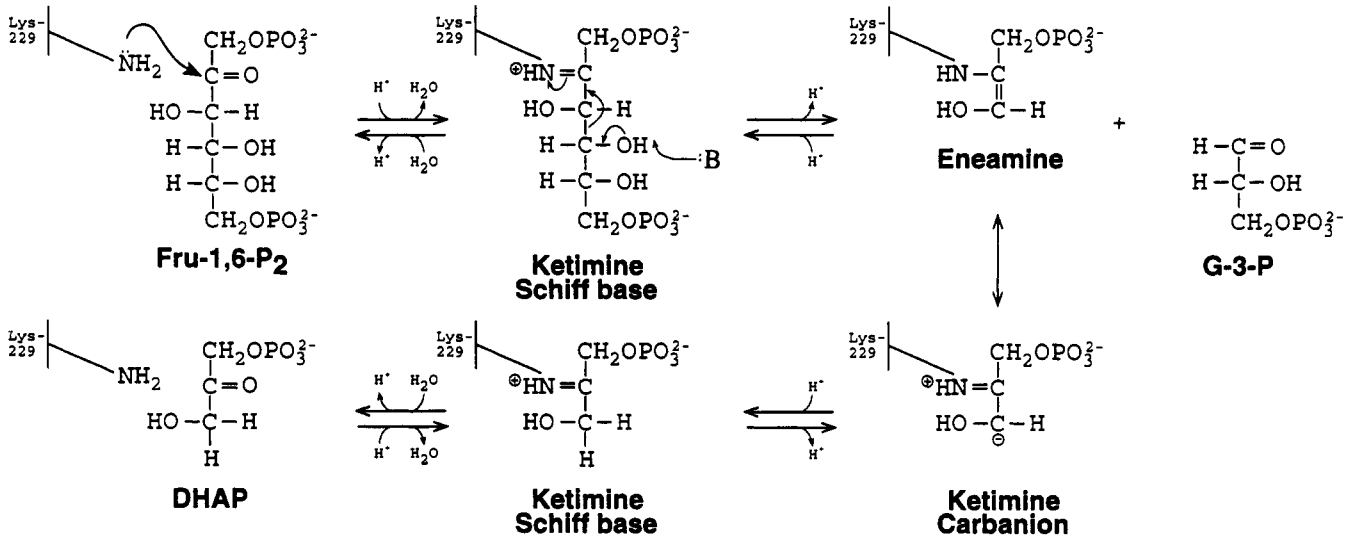


Table 3: Carbanion Oxidation Rates for Wild-Type and Mutant Enzyme–Substrate Complexes

| aldolase | Fru-1,6-$P_2$ $k'$ ($min^{-1}$) | DHAP $k'$ ($min^{-1}$) |
|---|---|---|
| wild type | 12.18 ± 0.57 | 12.67 ± 0.57 |
| K146A | 0.04 ± 0.05 | 0.09 ± 0.01 |
| K146Q | 0.02 ± 0.02 | 0.17 ± 0.02 |
| K146L | 0.03 ± 0.03 | 1.6 ± 0.09 |
| K146H | 0.03 ± 0.02 | 3.54 ± 0.09 |
| K229A | 0.07 ± 0.04 | 0.03 ± 0.04 |

panels B–D). This evidence, together with the finding that these mutants could form the carbanion intermediate only in the reverse direction, indicated that C3–C4 bond cleavage was the most drastically affected step.

For K146H, the rate of triose phosphate production ($k_2$) was measurable, although it was still considerably slower than the rate of Schiff base intermediate formation, as exhibited by the large $k_1$:$k_2$ ratio (Table 4). Furthermore, the value of $k_2$ was approximately the same as $k_{cat}$, indicating that this was the rate-determining step for this enzyme. For the wild-type enzyme, $k_2$ was approximately 9-fold greater than $k_{cat}$, indicating that the slow step was not C3–C4 bond cleavage but a step further along the reaction pathway (Scheme 1).

The activity of K146H was determined as a function of pH. The pH rate profile of the wild-type enzyme was flat over nearly the entire pH range tested, while the pH rate profile of K146H declined at pH > 7 (Figure 3). The drop in activity of K146H at pH > 7 is consistent with the rate-determining step for this enzyme being the acid–base chemistry required for C3–C4 bond cleavage. Furthermore, the titration of activity, with a p$K_a$ of approximately 8, may be due to the inserted histidine.

## DISCUSSION

Studies of the reaction pathway, discernment of reaction intermediates, and the kinetics of the individual steps of the aldolase reaction have been extensively investigated (Horecker et al., 1972; Creighton & Murthy, 1990). The slow step in the aldolase reaction has been difficult to determine unequivocally, and previous studies have concluded that it is either C3–C4 bond cleavage or release of triose phosphate products (Grazi & Trombetta, 1980; Rose & Warms, 1985; Rose et al., 1987). Most measurements of substrate cleavage depend on release of at least G-3-P from the enzyme; hence it is unclear whether one is measuring the rate of cleavage or the rate of product release. Acid quench directly measures the rate of C3–C4 bond cleavage, and not release, due to enzyme denaturation and subsequent release of products into solution. The data presented here on the rate of C3–C4 bond cleavage for the wild-type enzyme indirectly supports the conclusion by Rose et al. (1987) that DHAP release was the rate-determining step. The chemical step of C3–C4 bond cleavage is slow (6 $s^{-1}$ at 4 °C) but still 9 times faster than $k_{cat}$ at the same temperature (Table 4). Grazi and Trombetta (1980) measured the rate of C3–C4 bond cleavage indirectly and calculated a rate of 7 $s^{-1}$ at 22 °C, which was identical to $k_{cat}$. They concluded that C3–C4 bond cleavage was the rate limiting step. However, the rate of C3–C4 bond cleavage that they determined at 22 °C was nearly the same as the rate that we determined at 4 °C. This coincidence of rates is striking, since the difference in $k_{cat}$ at these two temperatures is 10-fold (Kuo & Rose, 1985).

The pre-steady-state chemical quench method depends upon the rate of denaturation being faster than the rates that are being determined, which has been discussed in previous reports that have used chemical trapping experiments (Grazi & Trombetta, 1978, 1980; Kuo & Rose, 1985; Rose & Warms, 1985). These reports concluded that for enzymes as slow as aldolase the denaturation step is relatively fast. Previous studies have primarily used trichloroacetic acid as the quenching agent (Grazi & Trombetta, 1980; Rose & Warms, 1985), but we found that HCl provided more efficient and reproducible trapping of covalent intermediates and that this acid did not interfere with TLC resolution of products released into solution. The optimal final concentration of HCl in the quenched reaction was 2 N, which is higher than the acid concentrations used by others (Kuo & Rose, 1985; Rose et al., 1987).

The pre-steady-state rate of Schiff base formation determined in this study was 140 times greater than $k_{cat}$ at 4 °C. Model et al. (1968) used $^{18}O$ exchange to measure the steady-state rate of Schiff base formation and found that it was only 4 times faster than $k_{cat}$. The acid quench method is thought to trap the Schiff base as well as the carbinolamine intermediates (Kuo & Rose, 1985). It is possible that the faster rates observed here reflect the rate of carbinolamine formation. However, interconversion of Schiff base and carbinolamine species is thought to be rapid (Rose et al., 1987), and therefore, differences in the rates of formation of these two intermediates seem an unlikely explanation of the discrepancy between the pre-steady-state rate of Schiff base formation and the steady-

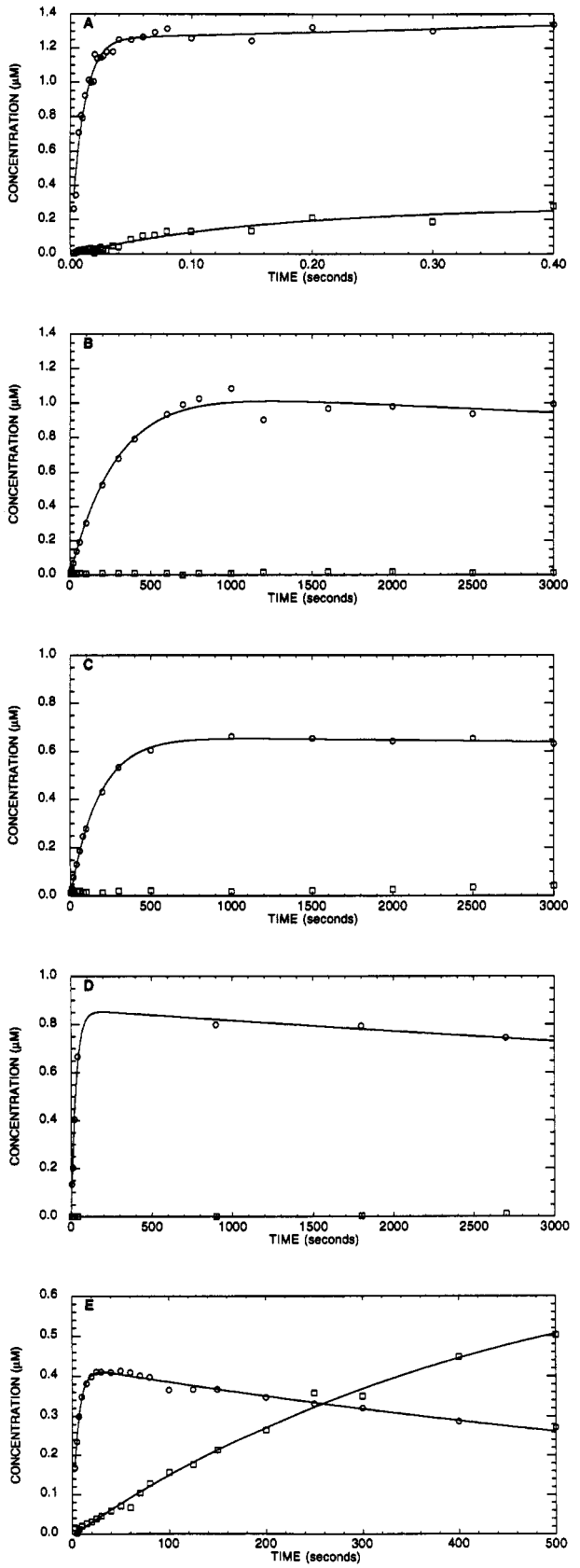


FIGURE 2: Single turnover in the forward reaction. A solution of [U-$^{14}$C]Fru-1,6-$P_2$ was mixed with a solution of enzyme to start the reaction, yielding final concentrations of 2.5 and 17.5 μM, respectively. The covalent intermediates (○), and triose phosphate products (□) were monitored. The curves were calculated as described in Materials and Methods: (A) Wild-type enzyme; (B) K146A; (C) K146Q; (D) K146L; (E) K146H.

state rate measured by exchange. The discrepancy is likely due to the effect of other reaction steps occurring in the steady state, both those involved in $k_{cat}$ and reversal of Schiff base formation. However, in the pre-steady state, the reversal of Schiff base formation could also be involved, which would tend to lower the measured rate of Schiff base formation, $k_1$, and lead to an underestimate of its value. Alternatively, the release of [$^{18}$O]water from the active site may be impaired. In fact, Littlechild and Watson (1993) proposed that this water molecule may be retained in the active site throughout the catalytic cycle.

*Role of Lysine-146.* For many years there have been attempts to identify the aldolase active site residues involved in catalysis (Horecker et al., 1972; Lai et al., 1974). Many of the conclusions of these studies have been discredited by analysis of the three-dimensional structure of aldolase (Sygusch et al., 1987) and comparison of the primary structures of different aldolases (Rottmann et al., 1984). One exception is the identification of Lys-146 as a potentially important residue (Hartman & Brown, 1976). They proposed that this residue may be involved in electrostatic binding of the phosphate groups of the substrates. Consistent with such a role for Lys-146 is its location in the active site. This lysine is 5 Å from the Schiff base forming Lys-229 (Sygusch et al., 1987). However, if Lys-146 were involved only in binding phosphate groups, substitution of Ala, Gln, or Leu would not have resulted in such profound loss of overall steady-state activity. This indicated a more essential role for Lys-146. Despite the drastic effect on $k_{cat}$, the mutant enzymes could form the carbanion intermediate from DHAP, yet they were unable to catalyze the next step in the reverse direction, namely, C3–C4 bond formation. Likewise, the mutants produced the critical Schiff base intermediate from Fru-1,6-$P_2$. Although the rate of Schiff base formation was $10^4$ slower than wild type, this did not explain the $10^6$-fold decrease in $k_{cat}$ (Table 2). Furthermore, the chemical trapping experiments failed to detect product[2] from the next step, C3–C4 bond cleavage (Figure 2 and Table 4). Therefore, in both directions of the reaction, the principal defect is clearly in the chemistry of C3–C4 bond cleavage and formation.

When Lys-146 was replaced by a histidine, considerable activity was recovered. The step affected in this mutant appears to be the same in both directions given that the effect on steady-state catalysis is the same in both cleavage and condensation. The inability to detect a carbanion with Fru-1,6-$P_2$ and the clear presence of this intermediate with DHAP (Table 3) indicated that the step leading to its formation from Fru-1,6-$P_2$ was slow relative to subsequent steps. The high $k_1$:$k_2$ ratio in the single-turnover experiments indicated that this slow step was not Schiff base formation but C3–C4 bond cleavage. The coincidence of $k_{cat}$ in steady-state assays with the rate of C3–C4 bond cleavage in pre-steady-state assays ($k_2$), along with all the aforementioned experiments, indicated that the replacement of lysine with histidine made C3–C4 bond cleavage the slow step. The pH profile indicated that a titratable group, possibly this histidine, was responsible for the activity of the enzyme below pH 8, provided the slow step of the enzyme does not change over this pH range. Furthermore, it appears that, in order to maintain catalysis, the residue at this position requires a positive charge and/or an ability to accept and donate protons.

Finally, Lys-146 is obviously a critical catalytic residue and is important for more than substrate binding. Littlechild & Watson (1993) proposed that the primary function of Lys-146 was in catalyzing Schiff base formation. The rate of Schiff base formation was reduced in all of the mutants, which indicates that Lys-146 may have some role in catalyzing this

Table 4: Rate Constants from Single-Turnover Experiments[a]

| | Fru-1,6-$P_2$–Schiff base formation | | triose phosphate production | | | |
|---|---|---|---|---|---|---|
| enzyme | $k_1$ ($s^{-1}$) | fold decrease from wild type | $k_2$ ($s^{-1}$) | fold decrease from wild type | $k_1$:$k_2$ | $k_{cat}$ ($s^{-1}$)[b] |
| wild type | 95 ± 5 | | 6 ± 1 | | 16 | 0.7 |
| K146A | 0.0035 ± (3 × $10^{-4}$) | 27 000 | c | | | 2.8 × $10^{-6}$ |
| K146Q | 0.0055 ± (2 × $10^{-4}$) | 17 000 | c | | | 6.6 × $10^{-6}$ |
| K146L | 0.033 ± (3 × $10^{-3}$) | 3000 | c | | | 9.2 × $10^{-6}$ |
| K146H | 0.171 ± (5 × $10^{-3}$) | 600 | 0.002 ± (3 × $10^{-4}$) | 3000 | 85 | 2.5 × $10^{-3}$ |
| K229A | c | | c | | | 1.8 × $10^{-6}$ |

[a] Wild-type experiments were at 4 °C, and mutants were at 21 °C. [b] $k_{cat}$ values were calculated from steady-state assays at 4 °C for wild-type aldolase and from the data in Table 2 for the mutant aldolases. [c] Not measurable.

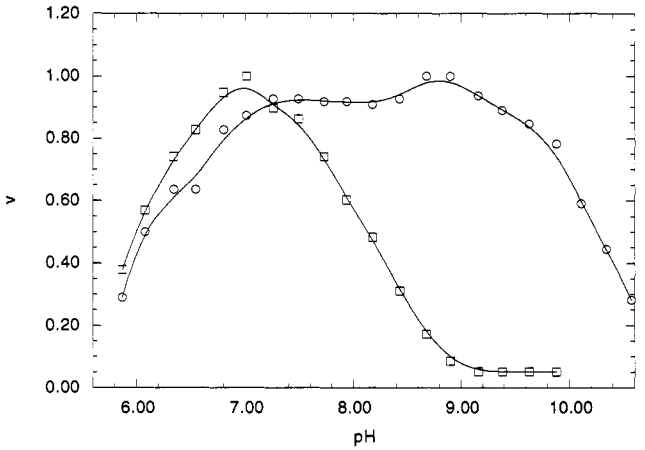


FIGURE 3: Relative velocity in the forward steady state versus pH. The pH dependence of catalysis is shown for wild type (○) and K146H (□).

step, possibly by lowering the p$K_a$ of Lys-229. However, an essential role for Lys-146 in this step seems unlikely in light of evidence presented here. Regardless of the substitution at position 146, the enzyme was able to form the Fru-1,6-$P_2$–Schiff base intermediate. It should be reiterated that the assay for Fru-1,6-$P_2$–Schiff base formation could have been measuring only carbinolamine formation, and therefore, it is possible that the inactivity of some of the Lys-146 mutants was due to a defect in their ability to dehydrate the carbinolamine. Yet, contrary to this explanation, all of the Lys-146 mutants were able to form the carbanion intermediate from DHAP, and this intermediate is formed after the Schiff base. Therefore, the primary defect in all of the Lys-146 mutants appears to be in cleavage of the C3–C4 bond of Fru-1,6-$P_2$. The positive charge of Lys-146 may aid in C3–C4 bond cleavage by stabilizing formation of the carbanion intermediate. Support for this hypothesis comes from the activity of the K146H mutant and the ability to titrate this activity. In addition, a K146R mutant also had partial activity (A. J. Morris and D. R. Tolan, unpublished data). Alternatively, Lys-146 may be the base that accepts the proton from the C4-hydroxyl of the substrate, or it may interact electrostatically with another residue, such as Asp-33, thus enhancing its ability to act as a catalytic base (Morris & Tolan, 1993).

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