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CYANIDE INACTIVATION AND PHOTOOXIDATION STUDIES.

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BOVINE LIVER 2-KETO-4-HYDROXYGLUTARATE ALDOLASE:
CYANIDE INACTIVATION AND PHOTOOXIDATION STUDIES

by

Barbara Ann Hansen

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1971

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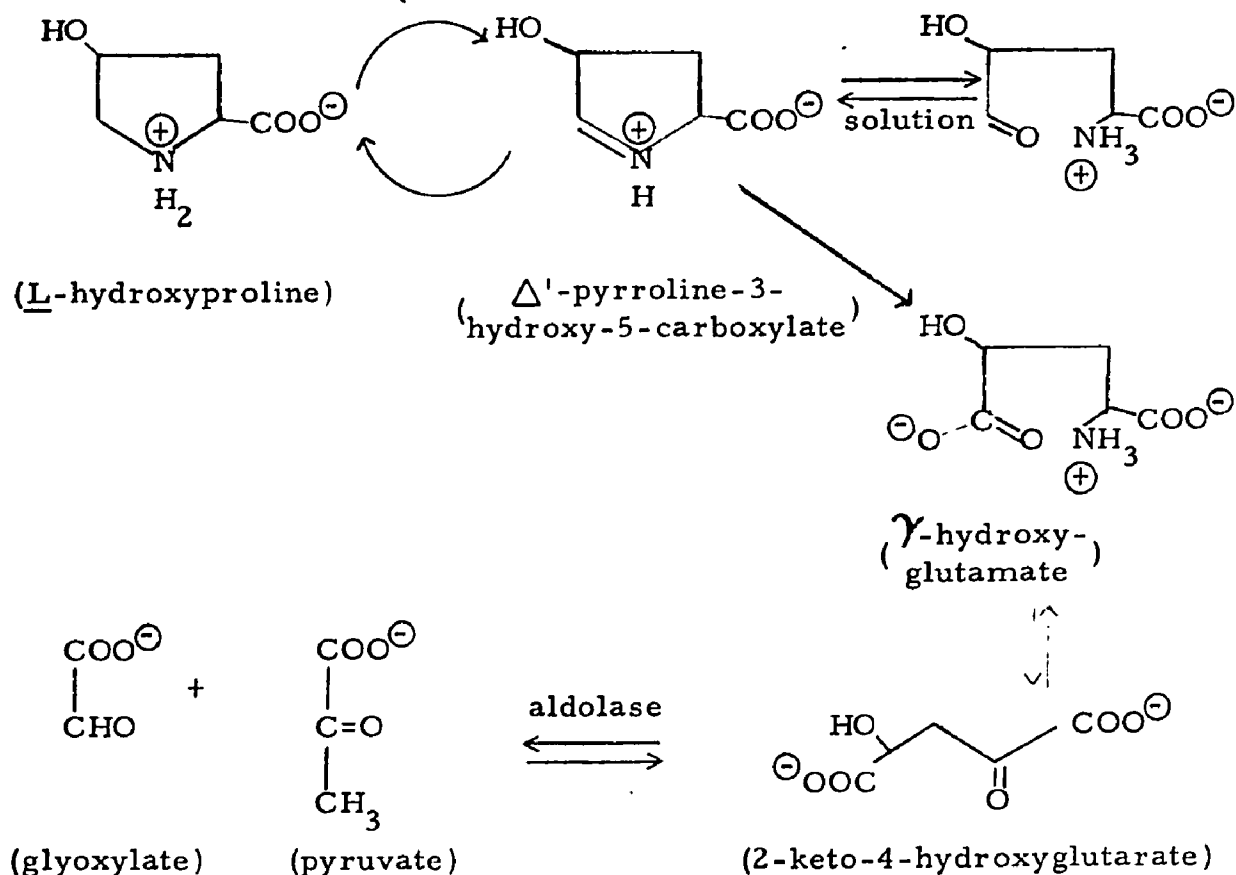
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ABBREVIATIONS

KHG	2-keto-4-hydroxyglutarate
KHB	2-keto-4-hydroxybutyrate
FDP	fructose 1,6-diphosphate
F-6-P	fructose-6-phosphate
Gly-3-P	glyceraldehyde-3-phosphate
DHAP	dihydroxyacetone phosphate
KDPG	2-keto-3-deoxy-5-phosphogluconate
OAA	oxaloacetic acid
HMP	4-hydroxy-4-methyl-2-pentanone
DRP	2-deoxy- <u>D</u> -ribose-5-phosphate
NADH	reduced nicotinamide adenine dinucleotide
LDH	lactate dehydrogenase
PMB	p-hydroxymercuribenzoate
TNM	tetranitromethane
DMSO	dimethylsulfoxide
SDS	sodium dodecyl sulfate
DEAE	diethylaminoethyl
Tris	tris(hydroxymethyl) aminomethane
CD	circular dichroism
IU	international unit of enzyme activity
<u>E. coli</u>	Escherichia coli
DTNB	5, 5'-dithiobis (2-nitrobenzoate)

INTRODUCTION

Since approximately 40 percent of total body protein consists of collagen-like extracellular protein, and L-hydroxyproline constitutes 13 percent of collagen, the metabolism and enzymology of this hydroxyimino acid is of central importance in animals. As late as 1960 the individual enzymatic steps for the breakdown of L-hydroxyproline were still unknown. Today, it is well established that 2-keto-4-hydroxyglutarate aldolase, the enzyme to be considered in this thesis, catalyzes a terminal step in the degradation of this imino acid in liver (1-5). The pertinent steps in this metabolic pathway are outlined below.



As can be seen, 2-keto-4-hydroxyglutarate (KHG) is the substrate in the final reaction; it is cleaved aldolytically yielding equimolar quantities of pyruvate and glyoxylate.

KHG-aldolase was first reported in the literature in 1960 when Kuratomi and Fukunaga (6) detected its presence in extracts of rat liver. In 1963, using 120-fold purified aldolase from rat liver acetone powder extracts, these investigators (7) described the following parameters for the enzyme-catalyzed reaction: a pH optimum of 7.4; an equilibrium constant of 0.73 (in phosphate buffer) favoring condensation of pyruvate with glyoxylate; K_m values of 1.0×10^{-2} M for pyruvate, 4.3×10^{-4} M for glyoxylate, and 1.33×10^{-3} M for KHG. A short time later, Maitra and Dekker (8) published a paper containing the values for many of the same constants determined with KHG-aldolase purified 60 to 70-fold from rat liver extracts. The pH optimum determined by these workers was 8.8 and the equilibrium constant 11.8 (in Tris·HCl buffer) favoring the cleavage rather than the condensation reaction. In addition, they provided evidence for a unique feature of this aldolase, namely, its lack of stereospecificity toward the two isomers of KHG. No other aldolase so far reported in the literature possesses this ability to utilize either optical antipode of its substrate. Because this work was done using a partially purified preparation of the enzyme, it was considered that the nonstereospecific character of the reaction might possibly be due to contaminating racemases. This was found not to be the case, however, when

Rosso and Adams (9,10) reported the same findings using KHG-aldolase purified 600 to 800-fold from rat liver acetone power extracts. This preparation of the enzyme approached homogeneity (3 or 4 trace proteins could still be detected) and the presence of a racemase seemed highly unlikely. It appeared that KHG-aldolase was truly nonstereospecific, catalyzing the cleavage or formation of either D- or L-KHG at the same rate and to the same extent. In addition, Rosso and Adams confirmed the values reported by Maitra and Dekker for the K_{eq} and pH optimum. In this same paper data pertaining to the mechanism of catalysis were first reported. Either ^{14}C -glyoxylate or ^{14}C -pyruvate was stably bound to KHG-aldolase in the presence of sodium borohydride concomitant with enzyme inactivation. After acid hydrolysis of the reduced complexes, the radioactive adducts were isolated and identified as their corresponding N^6 -lysyl derivatives, thereby demonstrating a possible involvement of Schiff-base intermediates in catalysis. Using a molecular weight of 120,000 which was determined by sucrose gradient centrifugation and by Sephadex column chromatography, Rosso and Adams calculated a value of 1 for the number of active sites per aldolase molecule; this value was based on the amount of radioactive substrate bound to the enzyme in the presence of borohydride. Finally, in 1969 Kobes and Dekker (11) achieved a 1300-fold purification of KHG-aldolase from extracts of bovine liver. This preparation was homogeneous as judged by starch gel, disc gel, and cellulose acetate strip

electrophoresis, and also by sedimentation patterns in the ultracentrifuge. Many of the properties of the beef liver enzyme were found to be identical to those determined for rat liver KHG-aldolase. Some general properties of bovine liver KHG-aldolase are summarized in Table 1.

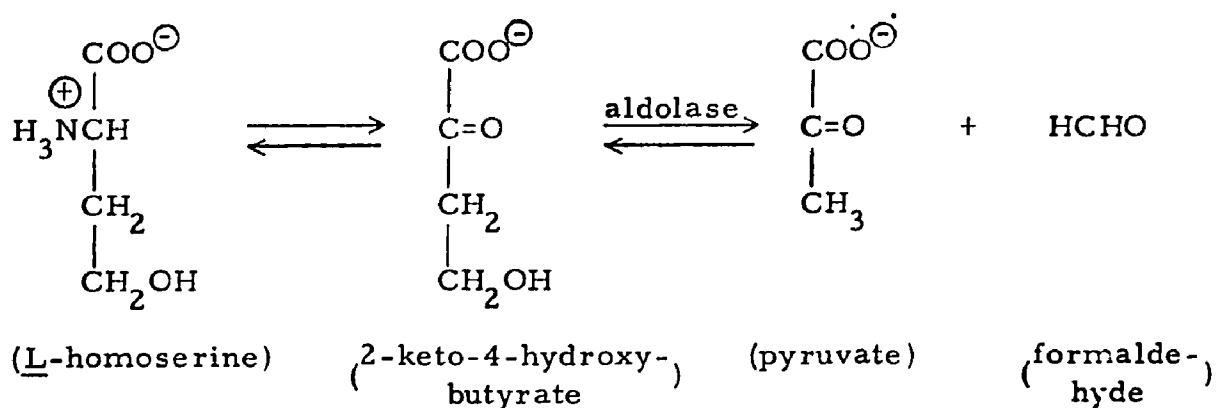
In addition to obtaining KHG-aldolase from rat and beef liver, this enzyme has also been purified from two bacterial sources. Dekker and Nishihara (12) purified KHG-aldolase to homogeneity from extracts of E. coli and found that it differed from the rat and beef liver enzymes in two respects; (a) its molecular weight is 62,000 to 64,000, approximately one-half that of mammalian KHG-aldolases, and (b) it does not possess the unusual ability to catalyze the cleavage of both optical isomers of KHG, but rather exhibits a strong preference (80%) for the L-isomer. KHG-aldolase has also been purified about 10-fold from extracts of an unidentified soil bacterium grown on 2-ketoglutarate as sole carbon source (13). The enzyme from this latter source, like those from mammalian liver, is nonstereospecific with respect to DL-KHG.

Lane and Dekker (14) reported in a recent paper that purified bovine liver KHG-aldolase also participates in the catabolism of L-homoserine.

Table 1

Some Properties of Bovine Liver KHG-aldolase

- (1) MW = 120,000
- (2) pI = 4.8
- (3) pH optimum = 8.8
- (4) $K_{eq} = 11.8$
- (5) Nonstereospecific with respect to
cleavage or formation of KHG
- (6) Schiff-base enzyme



In this pathway the enzyme catalyzes the aldolytic cleavage of 2-keto-4-hydroxybutyrate, a chemical analog of KHG, yielding stoichiometric quantities of pyruvate and formaldehyde. It is believed this is the same enzymatic activity as that reported by Hift and Mahler (15) where they observed the condensation of formaldehyde and pyruvate.

Classification of Aldolases

Aldolases have been divided into two general classes based upon their apparent catalytic properties (16). Class I aldolases are usually found in higher plants and animals, in algae, and in protozoa. One notable exception is KHG-aldolase (Class I) found in E. coli (12). These aldolases function in catalysis by a mechanism involving Schiff-base intermediates. They contain no bound metal ion, are not inhibited by various chelating agents, nor are they activated by the addition of mono or divalent cations.

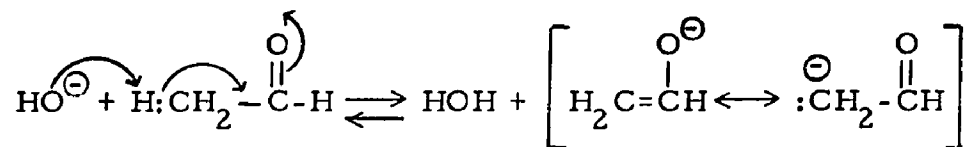
Class II aldolases, on the other hand, are found in bacteria, fungi, blue-green algae, and yeast; furthermore, they are activated by added metal ions and by $\text{K}^{\oplus}$. They apparently do not function via

a Schiff-base mechanism of catalysis but instead contain a metal ion (usually Zn^{++} , Fe^{++} , or Co^{++}) at the active site. It has been suggested that during catalysis this metal ion replaces the Schiff-base intermediate in the role of stabilizing the enolate-carbanion structure.

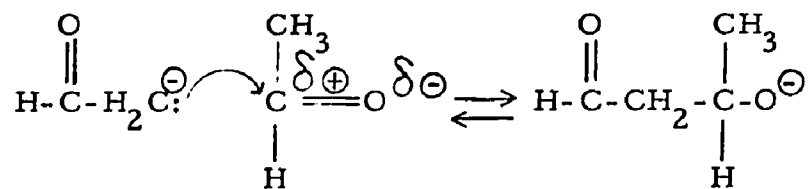
The work described in this thesis is concerned solely with Class I aldolases.

Chemically and Enzymatically Catalyzed Aldol Reactions

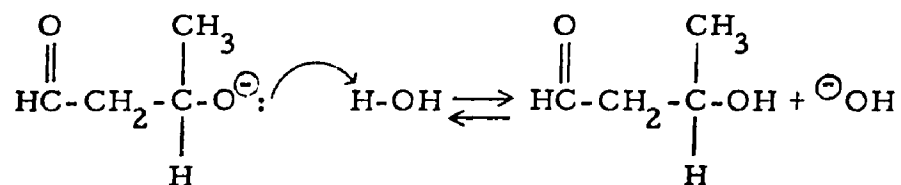
The aldol addition is specifically an addition of an enolate anion to a carbonyl double bond (17). The prototype of the chemical aldol reaction is the condensation of two moles of acetaldehyde to yield "aldol" or β -hydroxy-n-butyraldehyde. The first step in the base-catalyzed reaction is the generation of the enolate anion, as follows:



This anion then attacks the polarized carbonyl double bond of a second mole of acetaldehyde:

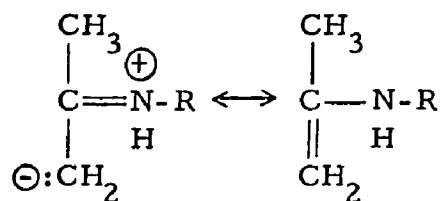


In the final step, the product picks up a proton from water regenerating the $\text{OH}^{\ominus}$ catalyst.



The equilibrium constant for aldol additions to aldehydes is usually favorable. The aldol addition to ketones is much less likely to occur, possibly because of steric hindrance or the greater stability of the carbonyl bond.

In 1938, it was reported by Westheimer and Cohen (18) that the aldol cleavage of 4-hydroxy-4-methyl-2-pentanone (HMP) was specifically catalyzed by hydroxide ion plus a primary or secondary (but not a tertiary) amine. They concluded that a Schiff base was being formed between the carbonyl of HMP and the amine. This type of complex would tend to disperse the negative charge formed during the aldol reaction by virtue of its ability to resonate between the ketimine and enamine forms:



A more sophisticated version of this same type of catalysis has been proposed for reactions catalyzed by Class I aldolases, the prototype of which is fructose 1,6-diphosphate aldolase from rabbit muscle. FDP-aldolase catalyzes the cleavage (formation) of D-fructose 1,6-diphosphate into (from) dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate. The mechanism of action of this

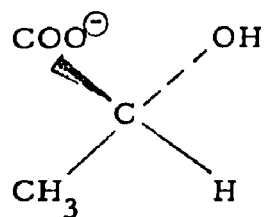
aldolase, as described by Rose et al. (19), contains the basic ingredients of the nonenzymatic catalysis described above; that is, an amine capable of Schiff-base formation and a general base catalyst.

Evidence for Base Catalysis of Aldol Cleavage or

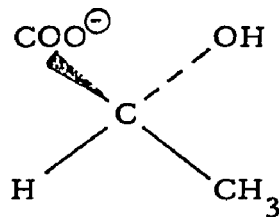
Condensation by Aldolases

The first concepts of aldolase catalysis portrayed the enzyme as a highly specialized basic catalyst also capable of providing an active surface for the reaction. The aldolase, like the hydroxyl ion in the nonenzymatic reaction, was presumed to abstract a proton from the carbon atom adjacent to the carbonyl (C-3 in DHAP) forming the enolate which then condensed with the carbonyl of the second substrate. This role of the enzyme was first given support in 1955 when Rose and Rieder (20) reported that rabbit muscle FDP-aldolase promoted the exchange of one atom of tritium from T_2O into its substrate, DHAP. This was confirmed by Bloom and Topper (21) and by Rutter and Ling (22). Because only one hydrogen was exchanged into DHAP in every case, it was concluded that this activation of DHAP was stereospecific. The stereospecificity of the reaction was rigorously established in 1958 by Rose (23) who isolated the tritiated DHAP, dephosphorylated it and oxidized it with periodate yielding tritiated glycolic acid. Oxidation of the glycolic acid to glyoxylic acid using glycolic acid oxidase, an enzyme which will also oxidize L-lactate but not D, revealed no release of tritium. It was concluded that the tritium in the glycolic acid was in one position only, namely, that

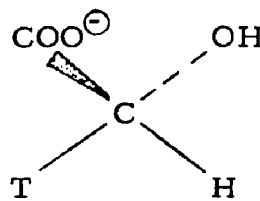
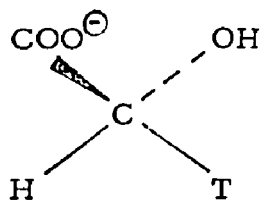
occupied by the α -proton in D-lactate. This can be seen by examining the structures below.



D-lactate



L-lactate

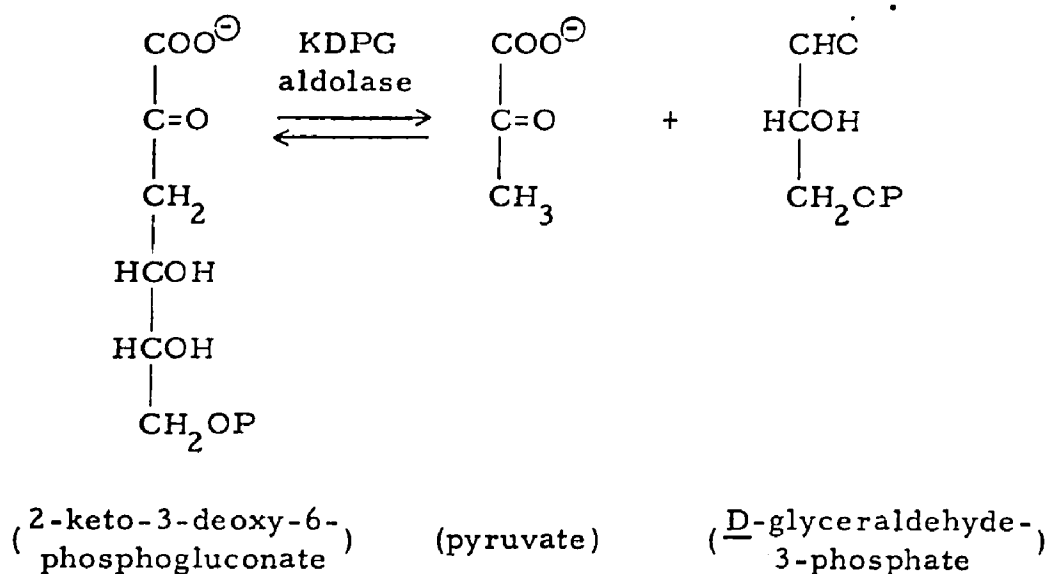


The tritiated glycolic acid.

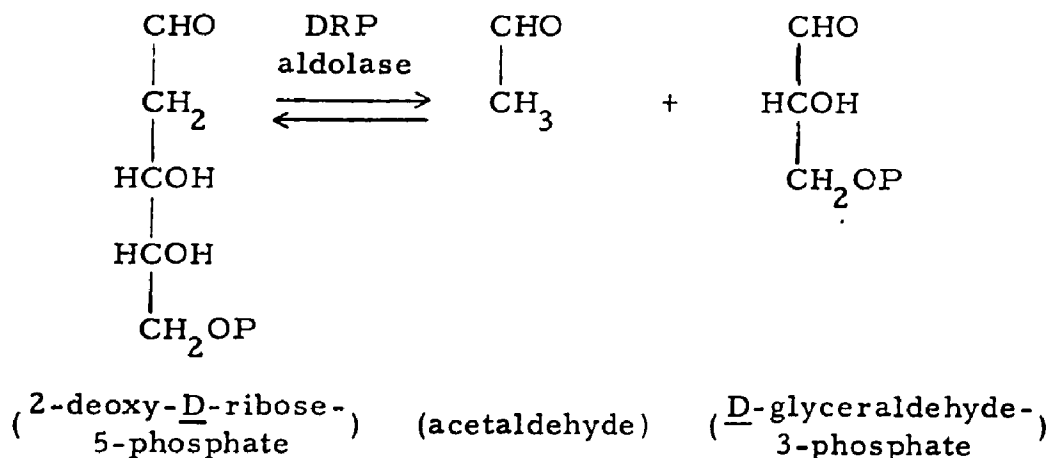
(the structure on the left corresponds to the glycolic acid produced in Rose's experiment)

Rose also found that the exchange reaction between T_2O and DHAP was inhibited by glyceraldehyde-3-phosphate as would be expected if the observed labilization of the α -proton of DHAP was an obligatory part of the enzyme catalyzed reaction, since glyceraldehyde-3-phosphate and the tritium would compete for the same site on DHAP.

This type of reaction has been demonstrated with other aldolases whose mechanisms of catalysis have proved to be analogous to that of FDP-aldolase. Examples are 2-keto-3-deoxy-6-phosphogluconate aldolase (24),



and 2-deoxy-D-ribose-5-phosphate aldolase (25).



It is of interest that a related enzyme, transaldolase, which catalyzes the transfer of the $\text{DHAP}^{\ominus}$ moiety from D-glyceraldehyde-3-phosphate in FDP to erythrose-4-phosphate forming sedoheptulose-7-phosphate, does not catalyze an exchange reaction with DHAP. Transaldolase forms a stable complex with $\text{DHAP}^{\ominus}$ which does not dissociate under normal conditions. The bound $\text{DHAP}^{\ominus}$ is only discharged by an acceptor aldehyde, and not by a proton as with the other aldolases.

The identity of the group(s) in the enzyme (protein) molecule which act as the base catalyst has been the subject of many protein

modification studies performed on various aldolases. The most extensive and conclusive of these were carried out using rabbit muscle FDP-aldolase. Of all the residues modified in this enzyme, only two appeared to be intimately related to the proton exchange reaction. These two residues were tyrosine and histidine. Modification of either residue destroyed the proton exchange capabilities of the aldolase without disturbing other steps in the reaction (e. g. binding of the substrate as evaluated by the determination of K_m values, or Schiff-base formation). The effect of tyrosine modification either by treatment with carboxy peptidase A (19,26-29), which removes 3 C-terminal tyrosyl residues, or by acetylation of tyrosine (30), is thought to be conformational in nature rather than due to destruction of the actual base catalyst. Many attempts to selectively modify the histidyl residues in FDP-aldolase have been made with various degrees of success. The most definitive of these was the photooxidation of rabbit muscle FDP-aldolase in the presence of pyridoxal phosphate as the photo sensitizer (31). In this paper, the authors reported the loss of 4 to 6 histidyl residues in FDP-aldolase, or approximately one residue per subunit (4 subunits per mole), following irradiation in the presence of pyridoxal phosphate. No other amino acyl residues were affected by this treatment. The resultant enzyme, which was 80 to 90% inactive, retained its ability to bind DHAP via a Schiff base, but did not catalyze proton exchange with DHAP. Aldol cleavage of FDP was stimulated by addition of the

acceptor aldehyde, erythrose-4-phosphate, providing strong evidence that the protonation of the Schiff-base anion is impaired and that the imidazolium ring of histidine is the group involved in this protonation process.

Evidence for a Schiff-base Intermediate in
Catalysis by Class I Aldolases

It was noted as early as 1955 (32) with the enzyme, transaldolase, that some type of stable intermediate was formed between the enzyme and the $\text{DHAP}^{\ominus}$ derived from fructose 1,6-diphosphate. This intermediate could transfer the $\text{DHAP}^{\ominus}$ moiety to appropriate acceptor aldehydes.

Later, Venkataraman and Racker (33) reported the actual isolation of this stable intermediate on a column of carboxy methyl cellulose. They confirmed earlier data indicating that the $\text{DHAP}^{\ominus}$ moiety could be transferred to an acceptor aldehyde. No speculation on the nature of the intermediate complex was offered.

The first reported spectrophotometric observation of an intermediate in FDP-aldolase catalyzed reactions appeared in 1957. Topper, Mehler, and Bloom (34) found that a mixture of rabbit muscle FDP-aldolase and DHAP absorbed light strongly in the ultraviolet region ($\lambda_{\text{max's}} = 233, 250 \text{ nm}$), as would be expected for an ene-diol/carbanion resonance structure. This observed absorbance occurred specifically with DHAP in combination with aldolase; other

proteins such as albumin or substrates such as D-glyceraldehyde-3-phosphate were ineffective. A significant reduction in absorbance could be achieved by adding D,L-glyceraldehyde-3-phosphate which presumably added to the activated DHAP[⊖] forming fructose 1,6-diphosphate. At the time this work was done, the catalytic intermediate was simply envisioned as an ene-diol stabilized in some way by the enzyme.

The exact nature of this intermediate was first deduced by Horecker and co-workers (35) who found that treatment of the transaldolase-DHAP[⊖] intermediate with sodium borohydride brought about a loss of enzymic activity and caused the irreversible binding of ¹⁴C-DHAP to the enzyme. Similar results were obtained using FDP-aldolase in combination with ¹⁴C-DHAP (36). In further publications, these authors provided concrete proof for the identity of the complex formed with either transaldolase or FDP-aldolase (37-39). After reduction of the ¹⁴C-DHAP-transaldolase complex with borohydride, the inactive enzyme was subjected to acid hydrolysis. The radioactive product was isolated and identified as N⁶-β-glyceryllysine, the N⁶-lysyl derivative of DHAP. A similar procedure carried out with ¹⁴C-DHAP and FDP-aldolase also yielded N⁶-β-glyceryllysine, indicating that with both enzymes the substrate is bound via a Schiff base to the ε-amino group of a lysyl residue (or residues).

Cyanide, a known inhibitor of Schiff-base enzymes, also was found to retard the activity of either FDP-aldolase (40) or

transaldolase (41) in the presence of the substrate, DHAP. This type of inhibition will be discussed in more detail later.

One further line of evidence for the participation of Schiff-base intermediates in enzymic reactions is the occurrence of an ^{18}O exchange process. This has been demonstrated by Model, Ponticervo, and Rittenberg (42) to occur with FDP-aldolase. The enzyme promotes the exchange of the carbonyl oxygen of FDP with ^{18}O from solvent H_2^{18}O as would be expected if the enzyme and its substrate formed a dissociable Schiff-base intermediate. This same type of ^{18}O -exchange phenomenon is observed with 2-keto-3-deoxy-6-phosphogluconate aldolase (43).

Some Unique Features of Enzyme-catalyzed Aldol Reactions

Enzymic catalysis of aldol reactions, as shown in Figure 1, has several features not possible in strictly chemical, nonenzymic ones, which enable the reaction to be selective and stereospecific with respect to reactants and proceed at greatly enhanced rates under physiological conditions. Three such features are; (a) the hydrophobic nature of the active site, (b) the presence of phosphate binding sites, and (c) the possibility of a fixed orientation of the amine and the general base catalyst. The primary sequence of the amino acids in the active site peptide of rabbit muscle FDP-aldolase indicates a highly hydrophobic environment (44). This binding has the following consequences:

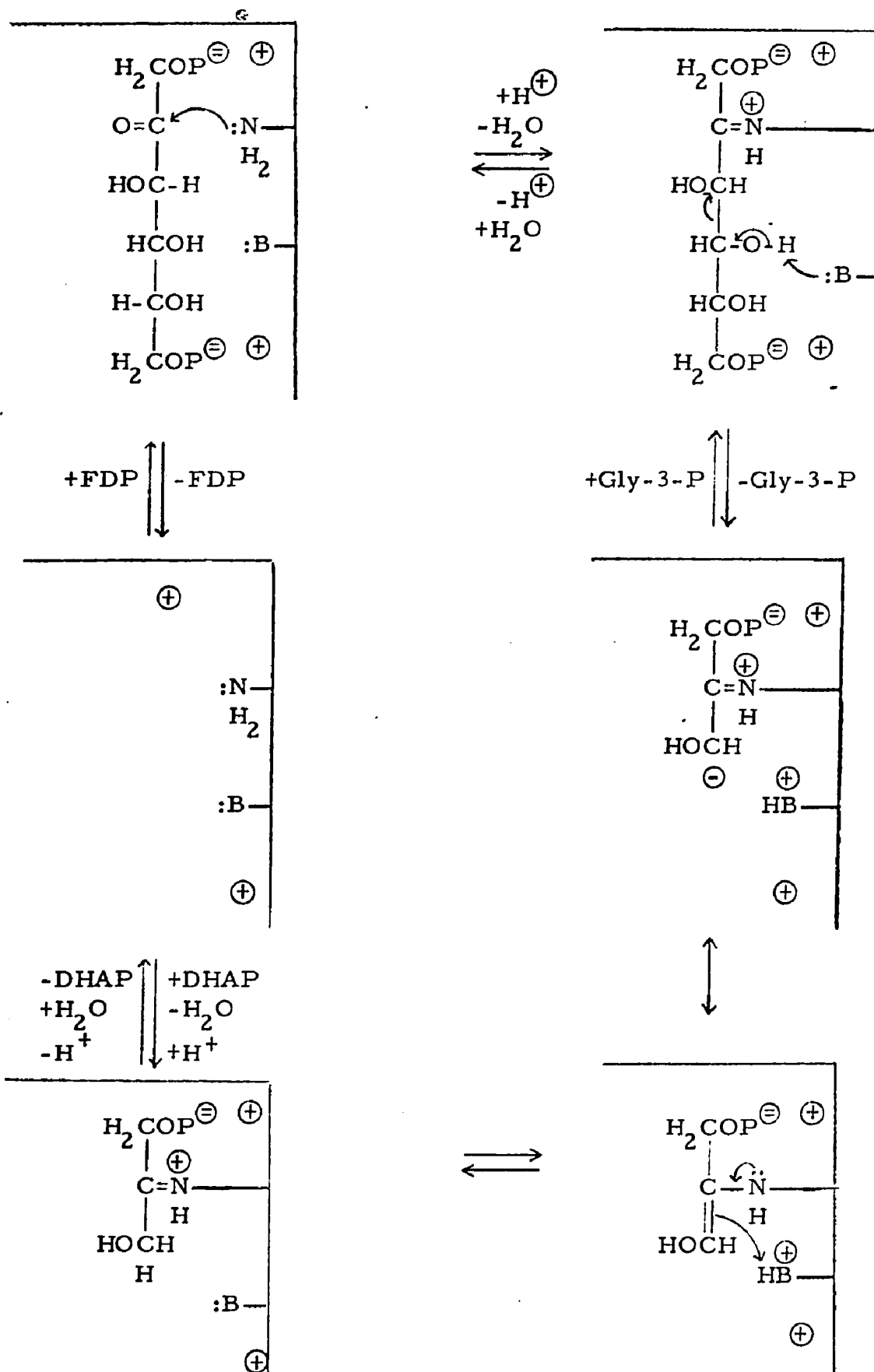


Figure 1. A Mechanism of Action for FDP-aldolase

- (1) The ϵ -amino group of the active site lysyl residue may be partially or wholly unprotonated even at pH values well under its normal pK_a around 10. It is the unprotonated form of the amine that is required for Schiff-base formation.
- (2) Schiff-base formation entails the elimination of a mole of water. Enhancement of Schiff-base formation would result from the rapid exclusion of this water from the hydrophobic active site.
- (3) The enamine-ketimine intermediate may be further stabilized by this hydrophobic environment by isolating it from solvent protons which would tend to discharge the complex.

The presence of clusters of positive charges in the protein molecule acting as phosphate binding sites is likely to be wholly responsible for the initial "recognition" and subsequent "binding" of the substrate prior to Schiff-base formation (45,46). Such "binding", in addition to its obvious potentiality for conferring substrate specificity, could orient the substrate in close proximity to the groups in the active site actually involved in catalysis, e.g., the amine and the base catalyst. The concentration of these catalysts in the reaction would thereby be effectively increased.

Finally, the fixed orientation of the amine and the ampholytes, $B:/B^{\oplus}H$, could impose more or less rigid requirements on the chemical and optical structure of the substrate.

The Mechanism of Action of Bovine Liver KHG-aldolase

Although KHG-aldolase has been discovered much more recently and has not been studied as extensively as has FDP-aldolase, several facts are known already about its mechanism of action. In many respects, it is similar to FDP-aldolase but it also possesses some unique characteristics. One of these has already been described, namely, its ability to utilize either optical antipode of KHG as substrate.

KHG-aldolase is a Class I (Schiff-base mechanism) enzyme; it is irreversibly inactivated in the presence of pyruvate and sodium borohydride. Unlike any other aldolase, however, KHG-aldolase is also inactivated in the presence of any of its other substrates (KHG, glyoxylate, or formaldehyde) and borohydride (47,48). Data obtained from competition experiments indicate that each individual substrate is bound by the same lysyl residue in the enzyme. For pyruvate, glyoxylate, and formaldehyde, the appropriate N^6 -lysyl derivatives have been isolated and identified after acid hydrolysis of the borohydride reduced enzyme-substrate complex. In each case, one mole of radioactive substrate is bound per mole (120,000 g) of aldolase.

KHG-aldolase also catalyzes an exchange process, promoting the incorporation of up to three atoms of tritium into pyruvate from a

T_2O/H_2O medium. Tritium exchange phenomena as catalyzed by KHG-aldolase are currently the subject of investigation in the laboratory in which this thesis research was carried out (49).

Studies by Kobes and Dekker (11) and Lane and Dekker (50, 51) on modification of the sulfhydryl groups of KHG-aldolase have established that such groups are required for enzymic activity. Titration of native KHG-aldolase with DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)) shows four accessible sulfhydryl groups which can be divided into two classes based on reactivity. One sulfhydryl group (Class I) titrates rapidly while three more (Class II) are modified at a slower rate. The first sulfhydryl group is titrated without loss of enzymic activity. Titration of the next three reduces enzymic activity to about 50% of its original value. Glyoxylate or pyruvate at 5 mM concentrations protect one Class II sulfhydryl group and 30 to 40% of the activity normally lost in the absence of substrate. It appears likely therefore, that at least one sulfhydryl group is in the active site of the enzyme and essential for complete catalytic activity. This conclusion is supported by studies in which KHG-aldolase was modified by reaction with tetranitromethane (TNM). With this reagent which modified only sulfhydryl groups (by oxidation), complete loss of enzymic activity was associated with the destruction of 4 sulfhydryl groups all of which were modified at the same rate. The loss of enzymic activity strictly paralleled the percentage of those 4 sulfhydryl groups that were destroyed. TNM-modified KHG-aldolase

was incapable of forming Schiff bases with its substrates as evidenced by its inability to stably bind radioactive pyruvate or glyoxylate in the presence of sodium borohydride.

An additional feature of KHG-aldolase is its ability to function as a β -decarboxylase with oxaloacetate as substrate (52). This finding is probably not too surprising in view of the fact that the enzyme, acetoacetate decarboxylase, functions via a Schiff-base mechanism almost identical to that of Class I aldolases. Bovine liver KHG-aldolase catalyzes the decarboxylation of oxaloacetate at about 50% the rate of KHG cleavage. KHG-aldolase from E. coli is actually a better decarboxylase as it catalyzes the loss of CO_2 from oxaloacetate at a rate roughly twice that of the cleavage of L-KHG (53). Substantial evidence indicates that the aldolase and β -decarboxylase activities of KHG-aldolase reside at the same active site on the enzyme. Another aldolase known to catalyze a decarboxylation reaction is KDPG-aldolase, which can also decarboxylate oxaloacetate but only at about 0.5% the rate of KDPG cleavage (54).

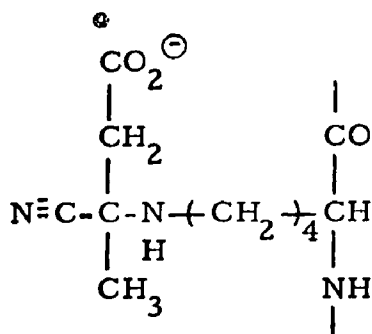
Cyanide as an Enzyme Inhibitor

Cyanide is known to be a versatile inhibitor of enzyme catalyzed reactions. One of its most toxic effects in mammals arises from its ability to combine with Fe^{+++} in cytochrome oxidase preventing reduction to Fe^{++} . In the same manner, cyanide is able to inhibit many metal ion-dependent enzymes by complexing the metal ion. Similarly, cyanide is also capable of reacting with methaemoglobin.

Some pyridoxal phosphate-requiring enzymes are inhibited by cyanide since it reacts with carbonyl groups forming cyanohydrins. For this reason, enzymes such as histidine decarboxylase from Lactobacillus 30a (55) and urocanase from Pseudomonas putida (56), which utilize pyruvate and α -ketobutyrate, respectively, as a prosthetic group, would also be expected to be subject to cyanide inhibition.

A slower, but virtually irreversible inactivation of certain enzymes may be achieved through scission of essential disulfide linkages by cyanide.

Another group of enzymes which have been found to be sensitive to cyanide are those functioning via Schiff-base mechanisms. The inhibition seen with these enzymes is dependent on the presence of a substrate capable of forming a Schiff base with the enzyme. The first report of this type of inhibition concerned acetoacetate decarboxylase (57-59). Fridovich, Westheimer and coworkers found that inhibition by cyanide occurred only in the presence of acetoacetate, acetone, or certain keto analogs. They proposed that cyanide was adding across the double bond of the Schiff-base intermediate in a manner similar to that described by Stewart and Li (60) for strictly chemical systems. The product of such an addition would be an enzyme-bound amino nitrile:



An enzyme species of this sort would be catalytically inactive.

Westheimer found, however, that this inhibition by cyanide was freely reversible precluding isolation of the adduct. At least two other enzymes which form Schiff-base intermediates are known to be subject to reversible inhibition by cyanide in the presence of a carbonyl substrate. These enzymes are transaldolase (41) and FDP-aldolase (40).

KHG-aldolase has been reported to undergo extensive inactivation by cyanide in the presence of either glyoxylate or formaldehyde (52). With this aldolase, however, the effect is irreversible even after extensive dilution and dialysis. This irreversible inactivation of KHG-aldolase by cyanide is the basis for part of the research work presented in this thesis.

Photooxidation as a Protein Modification Technique

It is well known that several amino acids are subject to modification upon irradiation in the presence of photochemical dyes (61). When these amino acids are incorporated into proteins, their reactivity in the process of photochemical oxidation is often changed enough so that in small proteins as few as 2 to 4 residues may be affected. Thus, Weil (62) found that photooxidation of α -chymotrypsin

in the presence of methylene blue destroyed one histidyl and three tryptophanyl residues concomitant with a 100% loss in enzymic activity. Since that time, methylene blue has been used to promote the photooxidation of amino acyl residues in many proteins. For enzymes with molecular weights of substantial size, however, photooxidation often destroyed significant numbers of amino acyl residues making definitive conclusions as to structure and function impossible. It did, however, provide in many cases an initial indication of amino acyl residues involved in catalytic activity.

In 1965, Westhead (63) reported the use of the dye, Rose Bengal, in the photochemical modification of yeast enolase. In certain cases, this dye appeared to be somewhat more selective than methylene blue. Rose Bengal was used quite successfully by Bond et al. (64) as the photosensitizer in the photooxidation of 3-phosphoglyceraldehyde dehydrogenase. This enzyme (MW = 140,000) is a tetramer containing four active sites. During photooxidation of the enzyme, 4 to 5 histidyl residues were destroyed coincident with a 50% loss of the initial enzymic activity. No other amino acyl residues were destroyed and the modified enzyme could still catalyze part of the over-all reaction, e. g. the binding of substrate. These investigators, therefore, were able to partially determine a role for histidyl residues in the protein molecule.

Horecker et al. (65) used this procedure with transaldolase (MW = 66,000) to specifically destroy a single histidyl residue and

achieve 90 to 100% inactivation of enzymic activity. The modified transaldolase was no longer able to form a Schiff base with fructose-6-phosphate. Results using Rose Bengal to photooxidize FDP-aldolase (MW = 140,000) were less definitive (66): About 70% of the 40 histidyl residues in the molecule were destroyed when 100% inactivation of catalysis was attained. The inactive aldolase was still capable of forming Schiff-base intermediates, although its ability to catalyze proton exchange was completely lost. It was, however, impossible to conclude whether the inactivation was due to the destruction of specific histidyl residues or merely to a conformational disruption of structure due to the destruction of so many amino acyl residues.

More recently, the use of active site-specific photosensitizers has become popular. Among the reagents used are porphyrins, flavins, and pyridoxal phosphate. Martinez-Carrion (67,68) reported the photooxidation of pig heart supernatant aspartate transaminase (MW = 100,000) using pyridoxal phosphate, its natural coenzyme. Out of 24 histidyl residues in the enzyme molecule, only 4 to 5 were destroyed in enzyme that was 80% photoinactivated. No other amino acyl residue was destroyed. The photooxidized enzyme could still bind the coenzyme, pyridoxal phosphate, as well as the keto or the amino acid substrate. The enzyme, however, was incapable of catalyzing the exchange of the α -hydrogen atom of the amino acid substrate with tritium from a T_2O/H_2O medium, as is required by

its mechanism of action. The authors, therefore, proposed the role of proton acceptor for the histidyl residues. Results obtained with FDP-aldolase, using the inhibitor, pyridoxal phosphate, as a photosensitizer have already been described.

Unfortunately, for many enzymes no active site directed photosensitizer is available and investigators must continue to rely upon less specific dyes. This is the case with KHG-aldolase as reported in this thesis. The dye, methylene blue, was determined to be the photosensitizer of choice, and although the results reported are not conclusive some new information about the aldolase has been uncovered.

MATERIALS

KHG-aldolase was purified from extracts of beef liver according to the procedure of Kobes and Dekker (11). Some preparations of the enzyme were further purified on a column of DEAE-cellulose through which a solution containing a diminishing concentration of ammonium sulfate was passed (69). The aldolase was applied to the column in 30% saturated ammonium sulfate solution and eluted with a gradient of 30 to 10% saturated ammonium sulfate solution in 0.025 M Tris·HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol. The KHG-aldolase used in these studies ranged in specific activity from 100 to 290. The best preparations available were used for substrate binding studies, for amino acid analysis, and in final PMB and DTNB-titration experiments.

D-KHG, L-KHG, and D, L-KHG were prepared from erythro- γ -hydroxy-L-glutamate, threo- γ -hydroxy-L-glutamate, and threo- γ -hydroxy-DL-glutamate, respectively, by nonenzymatic transamination, according to the procedure of Maitra and Dekker (5). KHB was synthesized in a similar manner from DL-homoserine, as described by Lane and Dekker (70). Formaldehyde solutions were prepared by acid hydrolysis of paraformaldehyde and subsequent distillation of the formaldehyde produced. Stock solutions of formaldehyde were standardized gravimetrically by precipitation with dimedon (71). Sodium glyoxylate was obtained from Sigma Chemical Co.; sodium pyruvate was a product of Nutritional Biochemical Corp.

The following substrate analogs were purchased from the sources listed: oxaloacetic acid and hydroxypyruvic acid (lithium salt) from Nutritional Biochemical Corp., methylglyoxal from Aldrich Chemical Co., acetaldehyde from Eastman Kodak Co., glycolaldehyde from Sigma Chemical Co., and glyoxal from Mann Research Labs.

^{14}C -Sodium cyanide (52.8 $\mu\text{c}/\mu\text{mole}$) was a product of Nuclear-Chicago Corp.; ^{14}C -formaldehyde (10 $\mu\text{c}/\mu\text{mole}$) was obtained from New England Nuclear Corp. The following radioactive compounds were all products of Amersham/Searle Corp.: ^{14}C -formaldehyde (17 $\mu\text{c}/\mu\text{mole}$), ^{14}C -sodium pyruvate (27.2 $\mu\text{c}/\mu\text{mole}$) and ^{14}C -sodium glyoxylate (16 $\mu\text{c}/\mu\text{mole}$).

Reagent grade sodium cyanide was obtained from Mallinckrodt Chemical Works; sodium borohydride was a product of Metal Hydrides, Inc.

N^6 -Carboxymethyllysine was a gift sample from Dr. Elijah Adams, The University of Maryland, Baltimore, Md. This compound was also synthesized chemically by reaction of α -N-carbobenzyl-L-lysine (Cyclo Chemical Corp.) with bromoacetate, according to the procedure of Hellerman (72). In a second chemical synthesis, N^6 -carboxymethyllysine was also prepared by alkylation of poly-L-lysine (Pilot Chemicals, Inc.) with iodoacetate, as described by Gundlach, Stein, and Moore (73). L-Lysine-HCl was purchased from Calbiochem.

Crystalline rabbit muscle lactate dehydrogenase (Grade A) was obtained in ammonium sulfate suspension from Calbiochem; its coenzyme, NADH, was purchased from P-L Biochemicals, Inc.

Methylene blue was a product of Frederick Smith Chemical Co. 2-Mercaptoethanol was procured from Matheson, Coleman and Bell, and glutathione from Nutritional Biochemical Corp.

The reagents used for quantitatively determining sulfhydryl groups, DTNB (5, 5'-dithiobis-(2-nitrobenzoate)) and PMB (p-hydroxy-mercuribenzoate), were obtained from Aldrich Chemical Co. and Sigma Chemical Co., respectively. Sodium dodecyl sulfate in "Ultra Pure" form was also a product of Sigma Chemical Co.

Constant boiling hydrochloric acid, packaged in 1 ml sealed ampoules, was used for acid hydrolysis of proteins prior to amino acid analysis. The HCl obtained in this form was purchased from Pierce Chemical Co. Dimethylsulfoxide, used in amino acid analysis procedures to convert all cysteinyl and cystinyl residues to cysteic acid, was a product of Baker Chemical Co.

METHODS

General Methods

The concentrations of proteins in solution were routinely determined by the method of Lowry et al. (74) using crystalline bovine serum albumin as a standard. An auxillary method used in certain cases to measure protein content of solutions was that of Murphy and Kies (75) which depends on peptide bond absorption at 215 and 225 nm. There was close agreement between these two methods when applied to the same solutions.

Radioactive compounds were detected on paper chromatograms using a Vanguard Autoscaner 880. Levels of radioactivity in solutions were determined on dried samples in planchets using a Nuclear-Chicago thin-window gas flow counter, which had an efficiency of about 32% for carbon-14. These measurements were also made by liquid scintillation counting techniques using a Packard Tri-Carb Scintillation Spectrometer Model 3320 with an efficiency of 73% for carbon-14.

Ultraviolet spectra were obtained with a Cary Model 15 recording spectrophotometer. Absorbance measurements at a set wavelength were made with a Gilford Model 2000 spectrophotometer equipped with a thermostated cuvette holder. Absorbance readings were either displayed on a digital readout meter or plotted with the aid of a Honeywell recorder. For certain assays involving the measurement of a product in the visible region of the spectrum, a

Klett-Summerson photoelectric colorimeter was used. Circular dichroism spectra were recorded on a Durrum-Jasco UV 5 scanning spectropolarimeter equipped to make CD measurements: the sensitivity was increased by a Sproul Scientific SS-20 CD modification.

Enzyme Assays

Two assays were available for the measurement of KHG-aldolase activity. The first, a single-point assay, involved the colorimetric determination of the glyoxylate formed during a 20 minute incubation of KHG-aldolase with its substrate, KHG, at 37°C. This assay has been described in detail by Maitra and Dekker (8).

The second is a rate assay in which the pyruvate formed by cleavage of KHG was utilized as the substrate for lactate dehydrogenase in the presence of NADH. The absorbance change at 340 nm was monitored. The assay mixture contained the following components: Tris·HCl buffer (100 μ moles, pH 8.4), NADH (0.33 μ mole), LDH (25 to 30 Iu), KHG (5 μ moles), and an appropriate amount of KHG-aldolase in a final volume of 1.0 ml. The addition of KHG-aldolase initiated the reaction; the temperature was maintained at 26°C.

The β -decarboxylase activity of KHG-aldolase was also followed by a coupled assay utilizing LDH. The assay mixture was incubated at 26°C and contained the following components: Tris·HCl buffer (100 μ moles, pH 8.0), NADH (0.438 μ mole), LDH (50 Iu), and an appropriate amount of KHG-aldolase. The reaction was started by the addition of OAA (3.3 μ moles); the total solution volume was

1.0 ml. The decrease in absorbance at 340 nm due to the oxidation of NADH was monitored. These assays were corrected by use of a control containing no aldolase which showed appreciable absorbance change due to nonenzymic decarboxylation of OAA.

Titration of Sulfhydryl Groups with DTNB and PMB

The procedure used to titrate sulfhydryl groups with DTNB was essentially that of Ellman (76), except that higher concentrations of DTNB and a slightly higher pH of reaction were used. A typical titration mixture contained KHG-aldolase (0.20 to 0.30 mg), Tris·HCl buffer (50 μ moles, pH 8.0), and DTNB (0.5 μ mole in 0.1 M potassium phosphate buffer, pH 7.0) in a final volume of 1.0 ml. The DTNB was added last and the change in absorbance at 412 nm, due to release of the 2-nitro-5-mercaptobenzoate anion, was monitored at 26°C. A molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ was used to calculate the number of sulfhydryl groups that reacted. A blank containing all components but no aldolase was used to correct for a small extraneous increase in absorption at that wavelength.

The titration of sulfhydryl groups in KHG-aldolase using PMB was followed spectrophotometrically, as described by Boyer (77) and further elaborated by Benesch and Benesch (78). This method takes advantage of an increase in absorbance at 250 nm accompanying mercaptide bond formation. Solutions of PMB were prepared by dissolving 10 mg of PMB (sodium salt) in 4 ml of 0.01 N sodium hydroxide solution. This solution was diluted to a volume of

25 ml with 0.01 M potassium phosphate buffer, pH 7.0, and stored in the dark at room temperature; it was stable for at least 2 weeks. The PMB solution was standardized by titrating a primary reference solution of glutathione, and double checked by spectral quantitation using a molar extinction coefficient of $16,900 \text{ M}^{-1} \text{ cm}^{-1}$ at 232 nm and pH 7.0. KHG-aldolase solutions prepared for titration with PMB contained 0.45 to 0.50 mg of the enzyme in 0.01 M Tris·HCl buffer, pH 7.4, in a total volume of 1.0 ml. The titration was carried out at 26°C by sequential addition of 5 μ l aliquots of the PMB solution (approximately 1 mM). After each addition, the contents of the cuvette were mixed and the absorbancy at 250 nm was measured. Aliquots of the PMB solution were usually added 15 to 20 μ l past the equivalence point. A control reaction mixture containing no aldolase was titrated in the same manner to correct for the absorbancy of the PMB itself at 250 nm.

Amino Acid Analyses

The samples of protein to be hydrolyzed were dialyzed exhaustively against 0.01 mM NaCl solution. After the protein content of the dialyzed solution was determined, an aliquot containing 1 to 2 mg of aldolase was pipetted into a constricted tube. An equal volume of constant boiling HCl was added. This solution was frozen and the tube then evacuated and flushed with nitrogen three times. The solution was then evacuated and thawed. The entire procedure was repeated three times more. After the final thawing, the solution

was again frozen, evacuated, and the tube sealed at the point of constriction. Hydrolysis was carried out at 110°C for 24 hours. At the end of this time, the sealed ampoule was opened, the contents quantitatively transferred to a round-bottomed flask, and the solution was concentrated to dryness at 40°C on a rotary film evaporator. The residue was dissolved in water and evaporated another three times as before. The final residue was dissolved in 1 to 2 ml of 0.2 M sodium citrate buffer, pH 2.2. Amino acid analyses were carried out with the use of an amino acid analyzer designed and constructed by Dr. Jules Shafer, Department of Biological Chemistry, The University of Michigan. Half cystine residues were determined after acid hydrolysis in the presence of dimethylsulfoxide, according to the procedure of Spencer and Wold (79). Tryptophan content was estimated spectrophotometrically on samples of unhydrolyzed enzyme, as described by Goodwin and Morton (80).

Photooxidation Studies

The process of photooxidation was carried out in a small tube (1.0 x 7.5 cm) partially immersed in a 250 ml beaker of water. The temperature of the water was maintained at 23°C by circulating water from a large reservoir into the beaker with a pump. For short periods of photooxidation (1 to 3 minutes), the temperature of the water remained constant without the use of the pump. The contents of the reaction tube were stirred by a micromagnetic stirring bar; the beaker and its contents were mounted on a magnetic stirring base.

Light from a 500-watt bulb was directed through the solution from a projector situated 20 cm away from the test tube. KHG-aldolase (0.2 to 3.0 mg) was introduced into the reaction tube with buffer and water to give a final volume of 0.5 to 1.0 ml. Methylene blue was added last and a sample withdrawn at zero time for determination of the initial enzymic activity. The lamp was then turned on and samples were removed at appropriate times for measurement of aldolase activity. At the end of the photooxidation process the lamp was turned off and a final sample withdrawn. A control tube, identical in composition to the reaction mixture, was always included. This sample remained in the dark for the duration of the experiment.

RESULTS

The Effects of Cyanide on KHG-aldolase

Reversible Inhibition of KHG-aldolase Activity by Cyanide

Figure 2 is a Lineweaver-Burk plot showing the effect of cyanide on KHG-aldolase activity. The observed inhibition is of a mixed competitive-noncompetitive nature. It is freely reversible upon dilution. The K_i for cyanide determined in 0.1 M Tris·HCl buffer, pH 8.4, by the method of Dixon (81) is 5.7×10^{-4} M.

Irreversible Inhibition of KHG-aldolase Activity by Cyanide in the Presence of Substrates and Substrate Analogs

In view of the fact that cyanide is known to have many and varied effects on enzyme-catalyzed reactions, it is not surprising that this substance interferes with KHG-aldolase activity in more than one way. In addition to being a mixed type, reversible inhibitor of aldolase activity, cyanide also causes irreversible inactivation of the enzyme in the presence of glyoxylate, formaldehyde, or other carbonyl compounds. This was shown in the following experiment:

Aldolase (0.22 mg), a substrate or a substrate analog (2.25 μ moles), and potassium phosphate buffer (50 μ moles, pH 7.4) in a volume of 0.26 ml were incubated for 10 minutes at 37°C. Cyanide (2.4 μ moles) was then added (final volume = 0.30 ml) and the mixture was incubated for an additional 30 minutes at 25°C. The protein was precipitated with 168 mg of ammonium sulfate and removed by centrifugation (12,000 $\times$ g). The pellet was washed twice in 80%

Legend to Figure 2.

Double reciprocal plots of KHG-aldolase activity as a function of KHG concentration in the presence of cyanide. Enzymatic activities were determined under standard assay conditions in 0.1 M Tris·HCl buffer, pH 8.4, and a 10 minute incubation period. In each assay 700 ng of KHG-aldolase were used. Initial velocities (v) are expressed as the number of μ moles of glyoxylate formed in 20 minutes at 37°C.

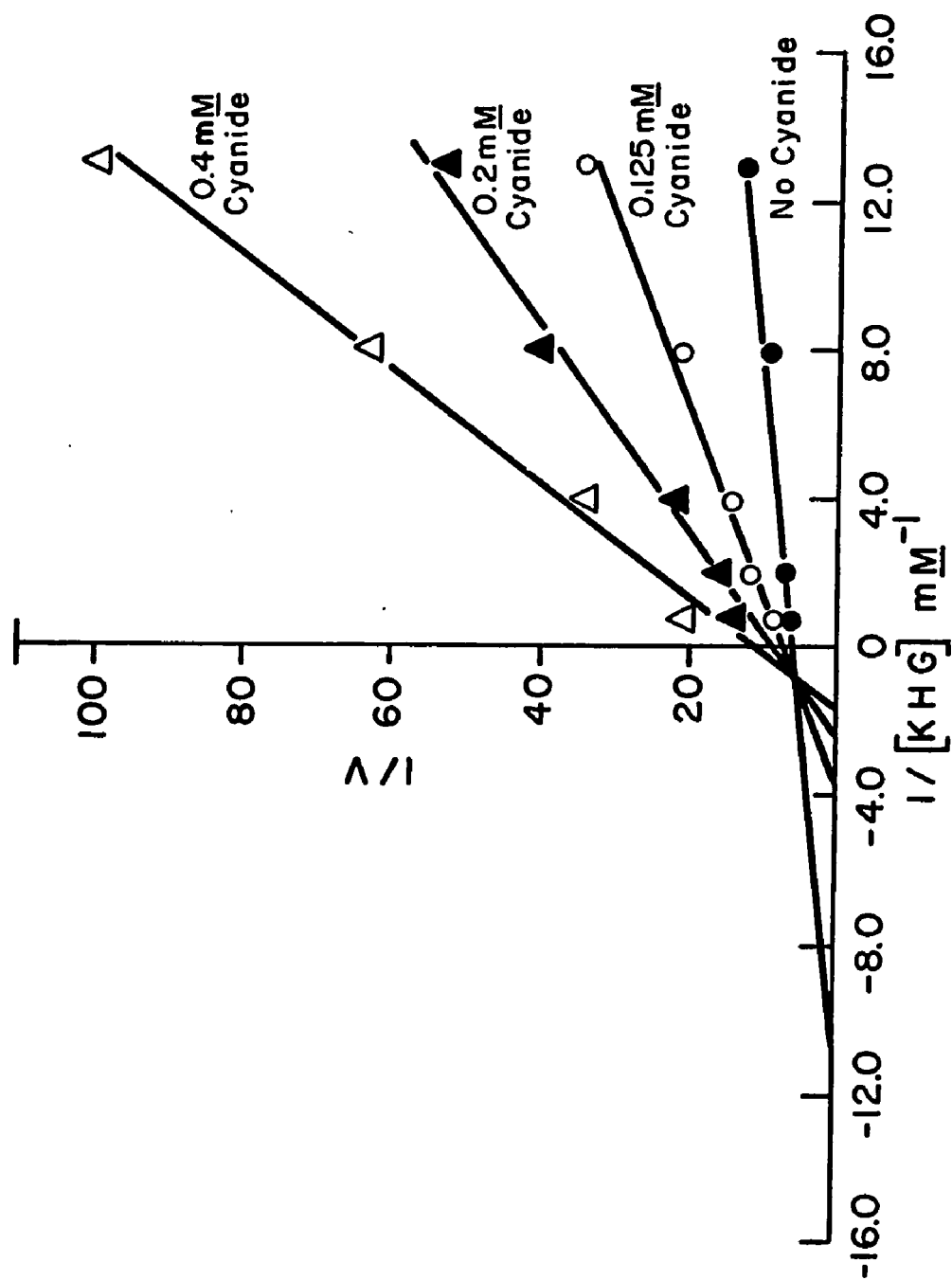


Figure 2. Reversible Inhibition of KHG-Aldolase by Cyanide

saturated ammonium sulfate solution and then dissolved in 0.30 ml of a mixture containing 0.05 M potassium phosphate buffer, pH 7.4, plus 5 mM 2-mercaptoethanol. Aliquots of the resulting solution were then assayed by standard procedures for aldolase activity and protein content. The results obtained with several substrates and substrate analogs are shown in Table 2. Cyanide alone does not cause irreversible inactivation of KHG-aldolase activity; it also is not effective in the presence of either KHG, KHB, or pyruvate. However, when the enzyme is incubated in the presence of cyanide and either glyoxylate or formaldehyde, 95% and 85%, respectively, of the initial enzymic activity is lost. Cyanide inhibition is also observed with a number of other carbonyl compounds. It is interesting to note that while a very limited effect is seen with pyruvate or acetaldehyde, their hydroxy analogs, hydroxypyruvate and glycolaldehyde, support an 86% and 70% inactivation, respectively, in the presence of cyanide.

Stoichiometry of Substrate Binding in the Presence of Cyanide

It has been shown that all three substrates (KHG, pyruvate, and glyoxylate) as well as various substrate analogs are bound reductively to KHG-aldolase when the respective enzyme-substrate complex is treated with sodium borohydride (48). In every case, the moles of substrate bound per mole of enzyme were found to be 1:1. Because it has been postulated with other enzymes which function via a Schiff-base mechanism that inactivation by cyanide is due to the

Table 2

Irreversible Inactivation of KHG-aldolase Activity by Cyanide
in the Presence of Substrates and Substrate Analogs

Substrate or Substrate Analog	% Inactivation
Cyanide alone	0
Cyanide plus:	
2-keto-4-hydroxyglutarate *	0
2-keto-4-hydroxybutyrate *	0
pyruvate	10
glyoxylate	95
formaldehyde	85
hydroxypyruvate	86
acetaldehyde	20
glycolaldehyde	70
glyoxal	75
oxaloacetate	17
methylglyoxal	30

* Incubations were carried out at 0°C, pH 6.2, to prevent dealdolization. Under these conditions, glyoxylate still caused its usual level of 90 to 100% inhibition of KHG-aldolase activity.

formation of an aminonitrile by addition of cyanide to the Schiff-base intermediate, it was of interest to determine whether the irreversible inactivation of KHG-aldolase activity by cyanide could provide some stoichiometric data. Table 3 shows the results of experiments involving the use of either ^{14}C -formaldehyde or ^{14}C -sodium cyanide. Neither of these two radioactive compounds alone causes loss of enzymic activity nor stable binding of radioactivity by the enzyme. However, when the aldolase is incubated with either ^{14}C -formaldehyde and unlabeled sodium cyanide, or ^{14}C -sodium cyanide and unlabeled formaldehyde, the enzyme is inactivated 75 to 85% and radioactivity is bound to the protein. From the amount of radioactivity bound, it was calculated that one mole of cyanide or of formaldehyde was incorporated per mole (120,000 gm) of inactivated enzyme. These results are consistent with the formation of a stable aminonitrile between KHG-aldolase, formaldehyde, and cyanide. Analogous results have been reported for KHG-aldolase in combination with glyoxylate and cyanide (52).

Identification of the Aminonitrile Formed with KHG-aldolase,

Formaldehyde, and Cyanide

Although aminonitrile formation is thought to be the basis for cyanide inactivation of several enzymes which function via Schiff-base mechanisms (40, 41, 58), in no case has this postulate been substantiated by the isolation and identification of such a product. Because of the unusual stability of the postulated aminonitrile formed with

Legend to Table 3

The reaction mixtures contained 50 μ moles of Tris·HCl buffer, pH 7.4, 250 μ gm of KHG-aldolase, and 144 μ moles of ^{14}C -formaldehyde (17 mc/mmole). These components were incubated for 10 minutes at 37°C and then 1.44 μ moles of sodium cyanide were added. The final reaction volume was 0.3 ml. In Experiment 4, unlabeled formaldehyde (1.44 μ moles) and ^{14}C -sodium cyanide (1.44 μ moles, 56.2 mc/mmole) were used. In either case, the mixture was incubated at 25°C for 30 minutes. The protein was precipitated by adding 168 mg of ammonium sulfate and subsequently removed by centrifugation at 12,000 x g. The pellet was washed twice with 80% saturated ammonium sulfate solution and then dissolved in 0.5 ml of 0.05 M Tris·HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol. This solution was dialyzed 24 hours against several changes (1 liter each) of the same buffer-mercaptoethanol mixture. Assays for protein content, KHG-aldolase activity, and for the level of radioactivity were run on the final dialyzed solution. From these data, the stoichiometry of binding was computed.

Table 3

Stoichiometry of Substrate Binding by KHG-aldolase
in the Presence of Cyanide

Experiment	Addition to Enzyme	Enzymic Activity Remaining	Radioactivity Bound	Substrate Bound
		%	cpm per μ mole protein	moles per 120,000 gm inactive protein
1	cyanide	100	0	0
2	^{14}C -formaldehyde	100	0.18×10^7	0.15
3	^{14}C -formaldehyde + cyanide	15	1.33×10^7	0.96^*
4	^{14}C -sodium cyanide + formaldehyde	25	4.78×10^7	1.01^{**}

* Corrected for the small amount of nonspecific binding of formaldehyde shown in Experiment 2.

** Enzyme treated with unlabeled NaCN in the presence of unlabeled sodium pyruvate then dialyzed exhaustively. This treatment was used to prevent nonspecific, stable incorporation of ^{14}C -cyanide into the enzyme.

KHG-aldolase (in contrast to the reversible inhibition in all previously noted cases), an attempt was made to isolate and characterize the adduct.

For this purpose, KHG-aldolase (0.25 mg) was incubated for 10 minutes at 37°C with ^{14}C -formaldehyde (1.44 μmoles , 17 mc/mmole) in Tris·HCl buffer, pH 7.4 (50 μmoles) in a total volume of 0.27 ml. Sodium cyanide (1.44 μmoles) was then added and the final solution (0.30 ml) was incubated 30 minutes at 25°C. After this interval of time, 83% inactivation of KHG-aldolase activity had occurred. The enzyme was precipitated with 168 mg of powdered ammonium sulfate and subsequently removed by centrifugation. The pellet was washed twice with 80% saturated ammonium sulfate solution and the final residue was then dissolved in 0.5 ml of 0.05 M Tris·HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol. The resulting solution was dialyzed 24 hours at 4°C against several changes (1 liter each) of the same buffer-mercaptoethanol mixture. Aliquots of the dialyzed solution were removed to determine the protein content and the amount of radioactivity present. As before, it was found that one mole of ^{14}C -formaldehyde was bound per mole of inactive enzyme. An equal volume of 12 N HCl was then added and the protein was hydrolyzed for 24 hours at 110°C in a sealed ampoule. The hydrolysate was evaporated to dryness in vacuo at 40°C. The residue was dissolved in water and the resulting solution applied to a column (0.5 x 5.0 cm) of Dowex 50(H^+) resin. The resin column was

first washed extensively with water and then eluted with 2 N ammonium hydroxide solution; fractions were collected. Those fractions containing radioactivity were pooled and this solution was evaporated to dryness in vacuo at 40°C. The residue was taken up in water adjusted to pH 6 to 7 and evaporated a final time. The final residue was dissolved in 0.15 ml of water. When subjected to paper chromatography, the radioactive product isolated by this procedure was found to migrate identically with authentic N⁶-carboxymethyllysine in five different solvent systems, as shown in Table 4. As best can be determined, this represents the first positive identification of an aminonitrile formed by reaction of cyanide with an enzyme-substrate Schiff-base intermediate.

Kinetics of the Irreversible Inactivation of KHG-aldolase Activity by Cyanide

Since the observed inactivation of KHG-aldolase activity by cyanide in the presence of formaldehyde (or other carbonyl analogs) is irreversible and can be followed by a rate assay, it was decided to examine the kinetics of this inactivation process. Kinetic data were obtained by coupling aldolase activity (which liberates pyruvate) with lactate dehydrogenase and measuring the disappearance of NADH at 340 nm. The rate of inactivation was followed after preincubating three different couples of the three component system (Figure 3). In each case, two of the three main components (aldolase, carbonyl compound, and cyanide) were put in the same solution and

Legend to Table 4

Whatman N° 3 filter paper was used with descending solvent flow for all chromatograms. Amino acids were detected by spraying with a 0.25% solution of ninhydrin in acetone. Radioactive compounds were detected using a Vanguard Autoscaner 880.

Legend to Figure 3

Semi-log plot of the percentage of initial KHG-aldolase activity as a function of time. Preincubation mixtures contained 50 μ moles of potassium phosphate buffer, pH 7.4, and two of the following three components: 300 μ gm of KHG-aldolase, 2.25 μ moles of glyoxal, and 4.4 μ moles of sodium cyanide. After 15 minutes preincubation at 37°C, the third component was added (T = 0). The volume of the final reaction mixture was 0.3 ml. At the times indicated, aliquots (2 to 5 μ l) of the reaction mixture were withdrawn and diluted into the LDH assay mixture.

Assay mixtures contained 100 μ moles of Tris·HCl buffer, pH 8.4; 5 μ moles of glutathione; 5 μ moles of KHG; 0.33 μ mole of NADH; and 60 μ gm of LDH in a total volume of 1.0 ml. The disappearance of NADH was followed at 340 nm.

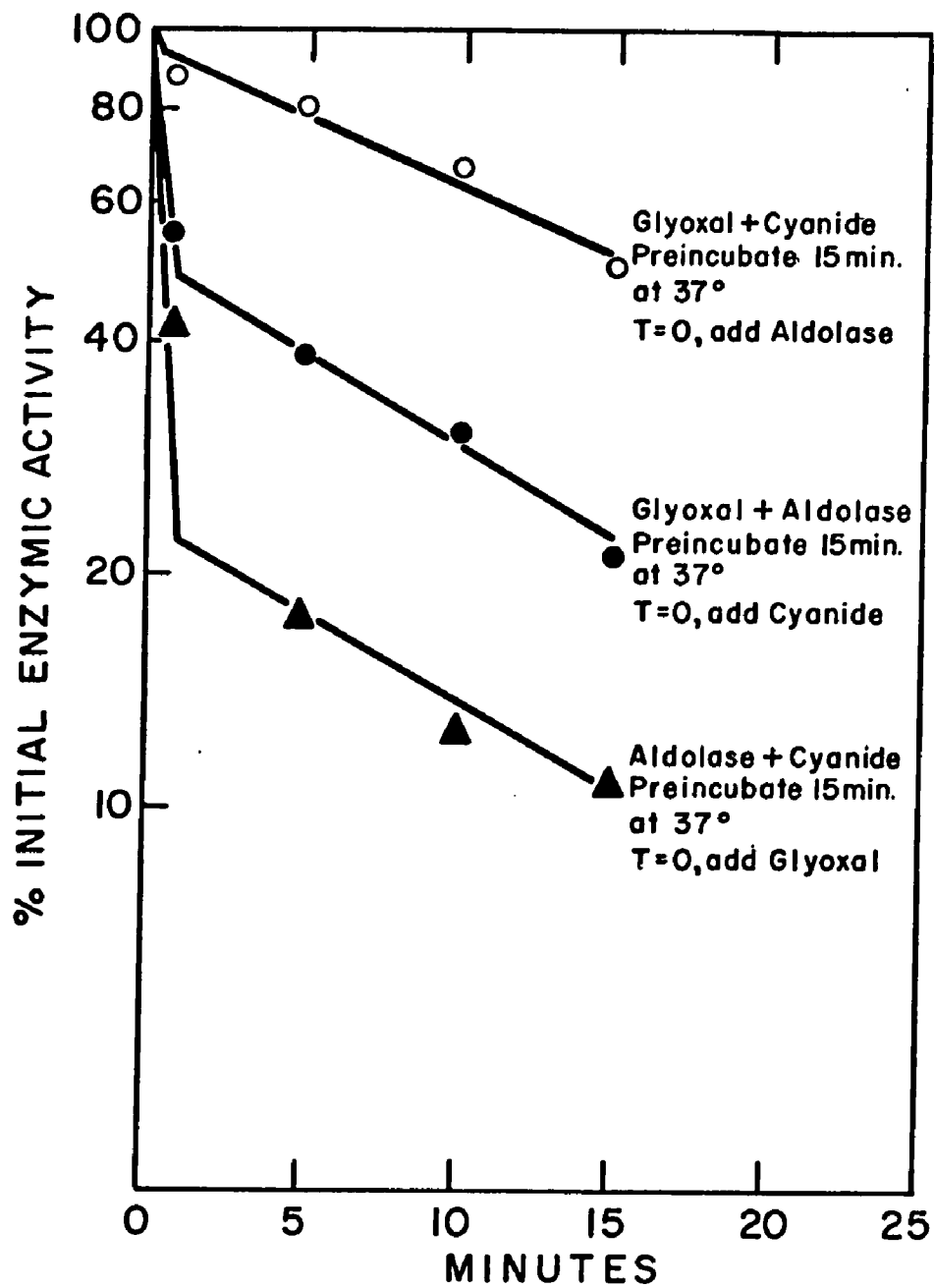


Figure 3. Inactivation of KHG-Aldolase by Cyanide in the Presence of Glyoxal

preincubated for 15 minutes at 37°C; the third component was then added at zero time ($T = 0$). Aliquots of the complete reaction mixture were withdrawn at the times indicated on the graphs and diluted ($\times 500$) into the LDH assay mixture. Although qualitatively similar data have been obtained using either formaldehyde, glyoxylate, or glycolaldehyde, the substrate analog, glyoxal, was used in these experiments because the rate of inactivation was relatively slower with this analog and hence the kinetics could be followed more conveniently.

As shown in Figure 3, the rate of inactivation is first order and biphasic. A possible explanation for results of this nature was first suggested by Cash and Wilson (40) and by Brand and Horecker (41). These investigators, observing reversible inhibition by cyanide of other Schiff-base mechanism enzymes, suggested that two possible equilibria might exist between cyanide, the enzyme, and the carbonyl substrate. The first of these equilibria was formation of the enzyme-substrate Schiff-base complex (which occurs rapidly) followed by the fast addition of cyanide to the azomethine linkage yielding an inactive aminonitrile. The second process was a reaction between cyanide and the carbonyl substrate with formation of a cyanohydrin; this latter reaction would (1) decrease the steady-state concentrations of free substrate and of the Schiff-base intermediate, (2) decrease the level of available cyanide, and (3) slowly release both cyanide and substrate into solution due to

dissociation of the cyanohydrin. If we assume the formation of cyanohydrin in this system is rapid (completed within one minute) and its breakdown is slow, this interpretation is consistent with the data shown in Figure 3. The middle line in this figure shows the result of preincubating glyoxal with KHG-aldolase and finally adding cyanide. The initial rapid rate of inactivation was tentatively attributed to the addition of cyanide to preformed enzyme-substrate complex (Schiff-base). The slow reaction then represents the rate-limiting breakdown of the cyanohydrin which releases more glyoxal and cyanide for reaction with the enzyme or the Schiff-base intermediate, respectively.

This interpretation is supported by the results plotted in the top line. In this experiment, glyoxal and cyanide were preincubated together allowing for cyanohydrin formation, and then KHG-aldolase was added at zero time. Under these conditions, no rapid rate of inactivation was observed; only the apparent slow, rate-limiting breakdown of cyanohydrin was detected.

It was expected that preincubation of cyanide with the aldolase followed by the final addition of glyoxal would give results somewhat similar to those shown by the middle line. Surprisingly the data shown on the bottom line were obtained. It seems likely in this case that a third reaction between the three components must be considered, namely, a reaction of cyanide with the enzyme which enables the enzyme to "hold" the cyanide in such a position that its

reaction with the Schiff-base intermediate, once formed, is unusually favorable.

The Effect of Preincubation Time on the Kinetics of Inactivation

In an effort to further test this model, the result of varying the time of preincubating two components together was observed in each of the three cases. Figure 4 shows data obtained in experiments in which the preincubation time was varied from 1/2 to 15 minutes. As expected, the data plotted in the first frame show that there is no apparent effect of time on the rapid equilibrium between enzyme, glyoxal, and the aldolase-glyoxal Schiff base. The middle frame shows the result of varying the time of preincubating glyoxal with cyanide. After 15 minutes preincubation time, a small degree of the rapid inactivation process is seen (about a 20% activity loss), but as the time is progressively shortened, somewhat more of the rapid inactivation reaction is seen each time until after only 1/2 minute almost 50% of the initial enzymic activity is lost in a rapid reaction. Although these results are qualitatively what would be expected, e. g. less and less cyanohydrin formed as the preincubation time is shortened, the cyanohydrin formation appears here to be much slower than would be consistent with the proposed scheme.

The frame on the far right in Figure 4 shows the result of varying the time of preincubating KHG-aldolase with cyanide. In this instance the result is opposite to that seen in the middle frame; more rapid inactivation reaction occurs after longer times of

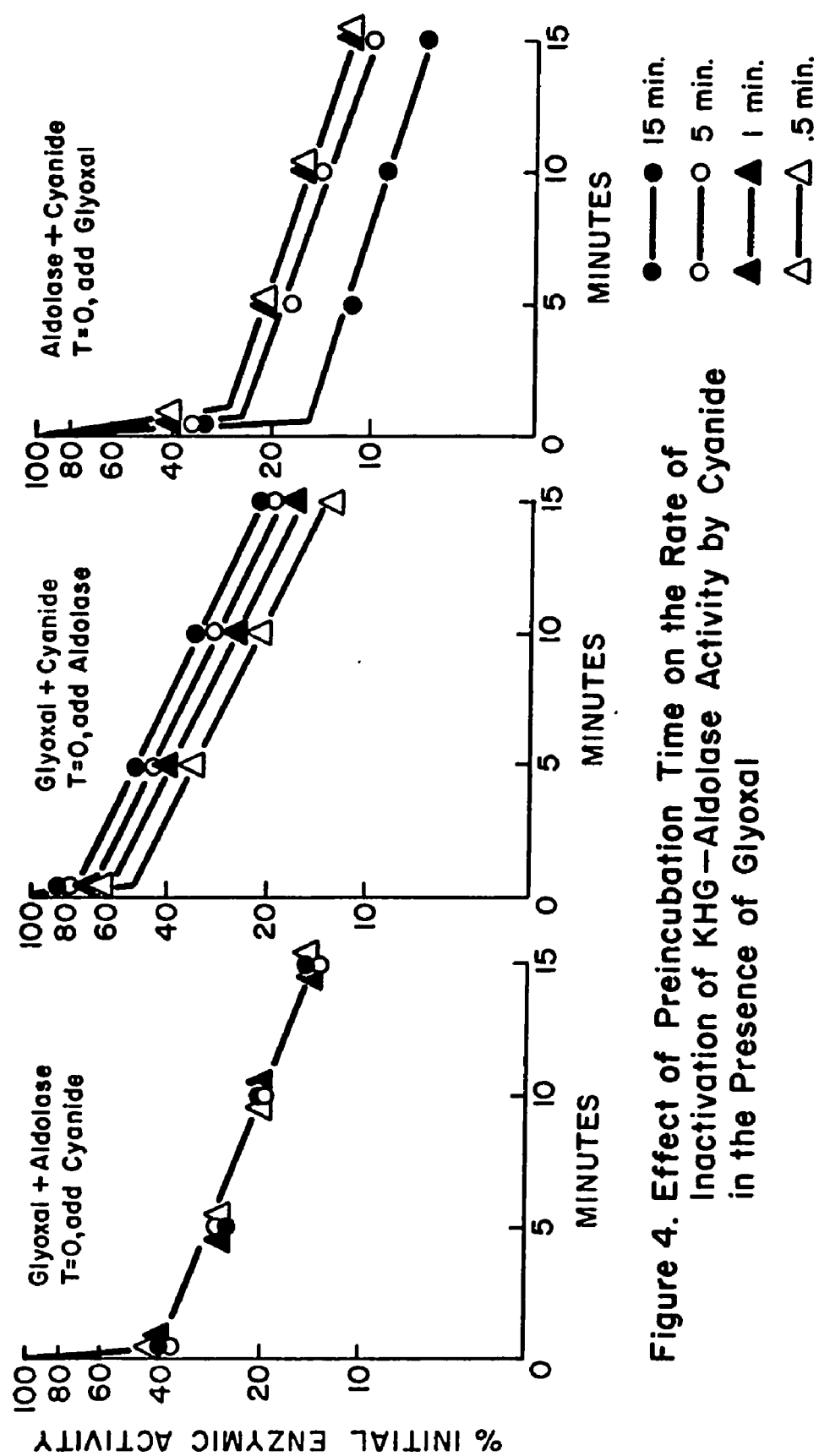


Figure 4. Effect of Preincubation Time on the Rate of Inactivation of KHG-Aldolase Activity by Cyanide in the Presence of Glyoxal

preincubation, although the actual change in this case is much smaller. The loss of initial enzymic activity after 1/2 minute of preincubation is about 70% in the rapid reaction as opposed to about 60% without any preincubation of cyanide and enzyme (Fig. 4, frame 1). After 15 minutes of preincubation time, this rapid inactivation reaction amounts to an 85% loss of the initial enzymic activity. These data also agree well with the proposed scheme. Although the reaction (or interaction) of cyanide with KHG-aldolase is slow, an effect can still be seen; longer preincubation times allow for more extensive reaction between the two components, thereby facilitating inactivation as soon as the Schiff-base intermediate is formed.

The Effect of Substrate Concentration on the Kinetics of Inactivation

According to the proposed scheme of competing equilibria, the limiting factor for the extent of the rapid inactivation reaction is the initial percentage of the aldolase existing as a Schiff-base intermediate. In each of the three cases, therefore, a higher concentration of glyoxal should cause more extensive rapid inactivation (as long as one is working below saturating levels of glyoxal). Figure 5 presents the results of an experiment which tests such reasoning. In every case, the prediction holds true; higher glyoxal concentrations cause more of the rapid inactivation reaction to occur. Note that when aldolase and cyanide are preincubated together (middle frame), the concentrations of glyoxal required to support extensive

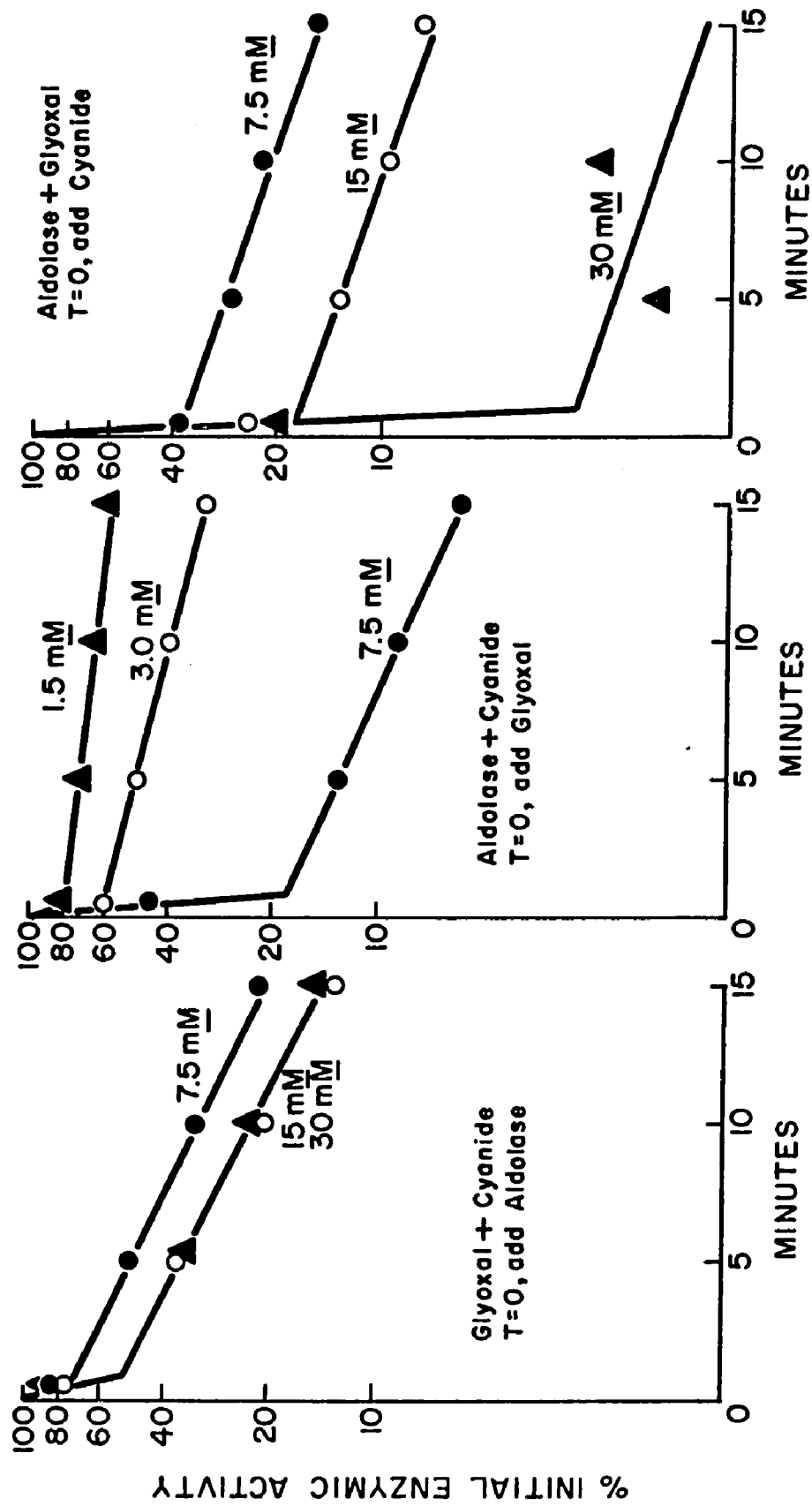
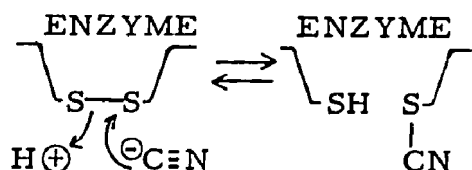


Figure 5. Effect of Substrate Concentration on the Inhibition of KHG—Aldolase Activity by Cyanide in the Presence of Glyoxal

rapid inactivation are significantly lower than in the other two cases. These results are consistent with a model in which the enzyme is able to "hold" the cyanide at or near the active site so that its reaction with the Schiff-base complex is greatly accelerated. Accelerated aminonitrile formation is thus able to pull the reaction between KHG-aldolase and glyoxal in the direction of Schiff base formation.

Nature of the Cyanide-aldolase Interaction: Sulfhydryl Content of KHG-aldolase Exposed to Cyanide

The kinetics of inactivation by cyanide in the presence of substrate suggest that cyanide is capable of interacting with the aldolase in some manner not dependent on substrate. The fact that cyanide is able to inhibit KHG-aldolase activity reversibly as a mixed-type inhibitor also implies an interaction between the enzyme and cyanide which is distinct from aminonitrile formation. Some possibilities as to what the nature of this interaction may be have been examined. The first possibility considered was the scission of a disulfide bond by cyanide; the reaction involved is the following:



The thiocyano portion of the cleaved disulfide bond may further react by internal cyclization rendering the reaction essentially irreversible (82). The new sulfhydryl group formed does not

undergo further reaction and therefore raises the sulfhydryl group titer of the enzyme by 1.

KHG-aldolase (0.525 mg), potassium phosphate buffer (100 μ moles, pH 7.4), and sodium cyanide (4.5 μ moles) were incubated for 15 minutes at 25°C in a total volume of 1.0 ml. The solution was then dialyzed exhaustively against three 1 liter volumes of 0.01 M Tris·HCl buffer, pH 8.0, containing no thiol. The dialysate was assayed for protein content and for the total number of free sulfhydryl groups in the enzyme molecule by DTNB titration in the presence of 1% sodium dodecyl sulfate. The results are presented in Table 5. There is no increase in the sulfhydryl titer of the aldolase exposed to cyanide; in fact, the cyanide-treated enzyme appears to have one less sulfhydryl group which is titratable with DTNB.

Nature of the Cyanide-aldolase Interaction: Cyanide Binding in the Absence of Substrate

The next possibility examined was to ascertain whether or not any cyanide might actually be associated with the aldolase in some manner and, if so, how much.

KHG-aldolase (0.797 mg), Tris·HCl buffer (200 μ moles, pH 7.4), and ¹⁴C-sodium cyanide (10 μ moles, 5.28 μ c/ μ mole) were incubated for 60 minutes at 25°C in a total volume of 1.0 ml. The solution was subsequently dialyzed against 0.01 M Tris·HCl buffer, pH 7.4, for 24 hours with 2 buffer changes (1 liter each). The

Table 5
Sulfhydryl Content of KHG-aldolase
after Exposure to Cyanide

Enzyme	Number of Sulfhydryl Groups Titratable by DTNB in 1% SDS
Native	10.1
Native, pretreated with cyanide	9.3
Native, pretreated with cyanide	8.8

dialyzed solution was assayed for KHG-aldolase activity, protein content, and the level of radioactivity present. The moles of ^{14}C -cyanide "bound" per mole (120,000 gm) of KHG-aldolase were computed; the results can be seen in Table 6. A measurable level of cyanide does, indeed, seem to be associated with the aldolase; the stoichiometry of this "binding" is about 0.4 to 1.0, cyanide to aldolase. The aldolase containing the "bound" cyanide is enzymically fully active. In separate experiments, the KHG-aldolase solution containing the "bound" ^{14}C -cyanide was then dialyzed again against either 1 liter of 0.01 M Tris·HCl buffer, pH 7.4, containing 10 mM unlabeled sodium cyanide or against 500 ml of 0.5 M KCl at pH 7.0. As also shown in Table 6, both of these treatments remove a significant amount of the radioactive cyanide initially associated with the enzyme. Similar results were obtained when either phosphate buffer, pH 7.4, or Tris·HCl buffer, pH 9.0, were used throughout the experiment.

Circular Dichroism Spectra of KHG-aldolase in the Presence and Absence of Cyanide

The possibility existed that cyanide interaction with KHG-aldolase resulted in a conformational change of either a gross or local nature. In order to investigate this possibility, circular dichroism spectra were recorded on KHG-aldolase in the presence and absence of 15 mM sodium cyanide. The CD cell contained KHG-aldolase (1.50 mg for the 250 to 350 nm region; 0.033 mg for the 200 to 250

Table 6

"Binding" of ^{14}C -Cyanide to KHG-aldolase
in the Absence of a Substrate

Additions to Enzyme	Further Treatment of Enzyme	Radioactivity "Bound"	umole ^{14}C -Cyanide "Bound" per Mole (120,000 gm) Aldolase
		cpm/umole protein	
^{14}C -Cyanide (10 mM)	none	3.41×10^6	0.40
^{14}C -Cyanide (10 mM)	KCl (0.5 M)	0.82×10^6	0.09
^{14}C -Cyanide (10 mM)	unlabeled NaCN (10 mM)	0.79×10^6	0.09

nm region), Tris·HCl buffer (100 μ moles, pH 7.4), and sodium cyanide (15 μ moles, adjusted to pH 8.0) in a total volume of 1.0 ml. Baselines were recorded on solutions containing all of the above components except aldolase. Spectra recorded in the absence of cyanide contained all of the other components in a total volume of 1.0 ml. The spectrum of native aldolase from 200 to 250 nm exhibited a double minimum at 210 and 220 nm indicating the presence of α -helical structure in KHG-aldolase. The spectrum recorded in 15 mM cyanide was identical to that of native aldolase indicating that no gross conformational changes are induced in the presence of cyanide.

The spectrum of native KHG-aldolase at higher wavelengths (250 to 350 nm) showed a maximum between 280 and 290 nm with a shoulder at 275 to 280 nm. In the presence of 15 mM cyanide, this peak, although of the same general shape, appeared to decrease in amplitude to approximately 70 to 80% of that observed with native enzyme. This region of the spectrum (275 to 290 nm) is attributed to tryptophan/tyrosine absorbance and a change in the CD spectrum in this range could possibly indicate a local shift of one or more of these aminoacyl residues in the intact protein molecule.

Photooxidation of KHG-aldolase in the Presence of Methylene Blue

The participation of an active site lysyl residue in the mechanism of action of KHG-aldolase has now been well established. Enzyme inactivation studies, already described, using sodium

borohydride (47) or cyanide (see earlier section of this thesis) to react with the enzyme-substrate complexes have shown the involvement of this lysyl residue in forming Schiff-base intermediates with the substrates. The second major catalytic event in the proposed mechanism of action of KHG-aldolase, namely, proton removal and donation, has yet to be examined. In other aldolases, the structure within the protein molecule responsible for this amphoteric behavior has been determined to be the imidazolium group of histidine. Because this particular residue is highly sensitive to dye-catalyzed photooxidation, this approach was tried with methylene blue and KHG-aldolase from bovine liver.

The Kinetics and pH Dependence of the Photoinactivation of KHG-aldolase

The time course of the photoinactivation process can be seen in Figure 6. The rate of inactivation, shown here at pH 8.0 in Tris·HCl buffer, is rapid and pseudo-first order to a point where at least 96% of the enzymic activity is lost. When the pseudo-first order rate constants are plotted as a function of pH, it can be seen in the inset that the rate of photoinactivation varies sigmoidally with the pH between the range of 6.0 and 9.0. An inflection point occurs at approximately pH 7.3. At pH values below 6.0, the enzyme precipitated during the course of photooxidation. A pH-rate profile similar to that shown in Figure 6 has been reported for the photooxidation of a solution of free histidine in the presence of

Legend to Figure 6

KHG-aldolase (1.62 mg), Tris·HCl buffer (100 μ moles, pH 8.0), and methylene blue (80 nmoles) were mixed in a total volume of 1.0 ml. This solution was irradiated for 15 minutes at 23°C, as described in the Methods section. Aliquots (5 μ l) were withdrawn at the times indicated and diluted (100-fold) into 0.05 M Tris·HCl buffer, pH 8.0, containing 5 mM 2-mercaptoethanol. The diluted aliquots were assayed for KHG-aldolase activity by the standard colorimetric estimation of glyoxylic acid. A control tube, identical in composition to the test solution, was kept in the dark and showed no loss of activity during the 15 minute interval of time.

Inset: KHG-aldolase (0.27 mg), buffer (50 μ moles), and methylene blue (20 nmoles) were mixed in a total volume of 0.5 ml. Aliquots (5 μ l) were withdrawn at appropriate times and diluted (100-fold) into 0.05 M Tris·HCl buffer, pH 8.0, containing 5 mM 2-mercaptoethanol. The diluted aliquots were tested for KHG-aldolase activity. The pseudo-first order rate constants were calculated from semi-logarithmic plots of the data. The following buffers were used at the pH values indicated: potassium phosphate, pH 6.0 to 7.5; Tris·HCl, pH 7.5 to 9.0. Some variation in rates of photo-oxidation between the two buffer systems was normalized by comparing the rate constants at pH 7.5 in both buffer systems.

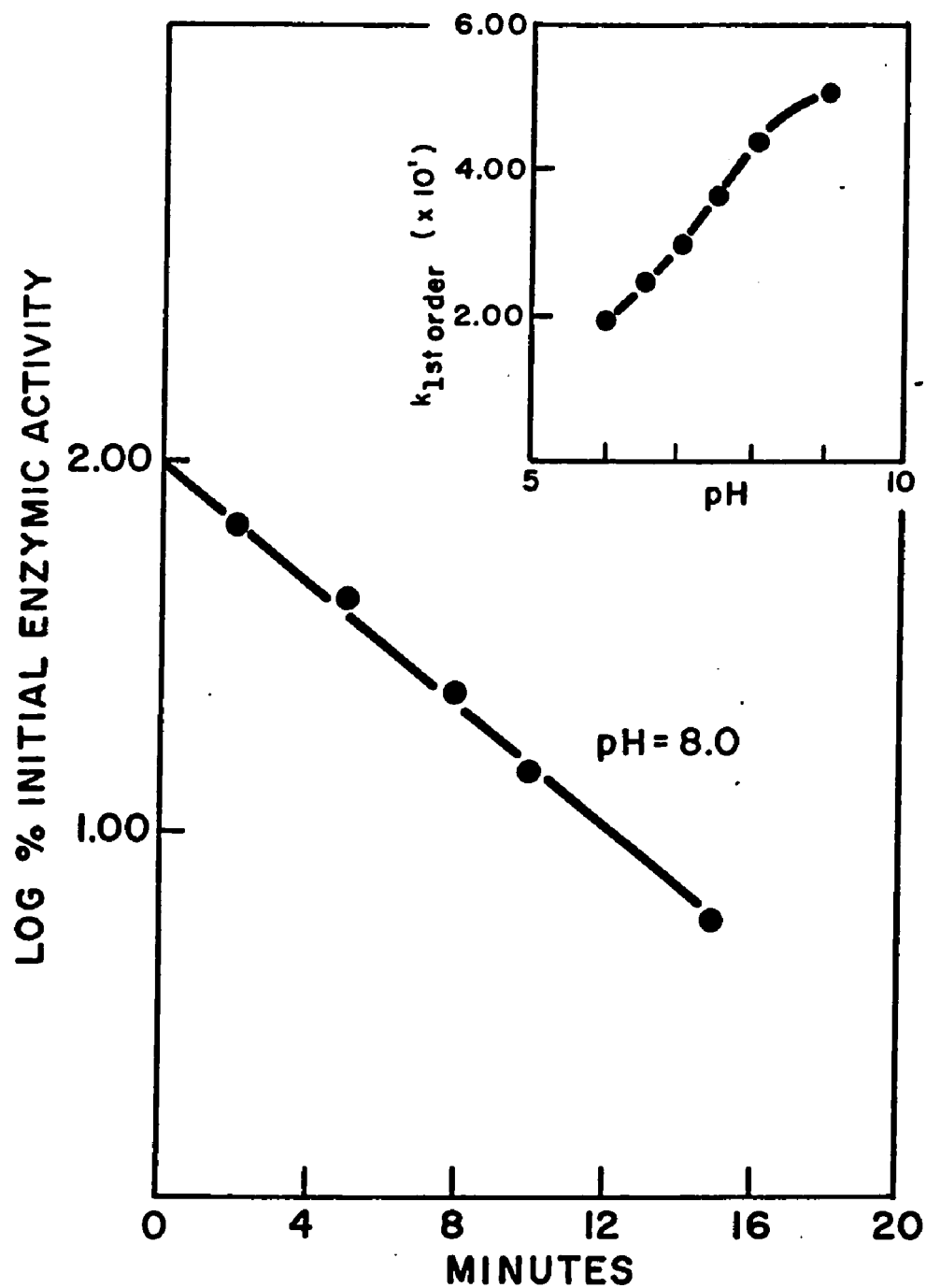


Figure 6. Photooxidation of KHG-Aldolase in the Presence of Methylene Blue

methylene blue (61), as well as for the photoinactivation of enzymes in which histidine was the sole amino acyl residue found to be destroyed by this treatment (83). As shown in Figure 7, however, this very same type of pH-rate profile is also obtained for the photooxidation of the sulfhydryl group of glutathione. The pH range extends only to 8.5 in this figure since precipitation occurs during photooxidation at higher pH values.

The Effect of Photooxidation on the β -Decarboxylase Activity of KHG-aldolase

Ample evidence indicates that the same active site(s) of this enzyme is involved in both its β -decarboxylase activity toward OAA and its ability to catalyze the aldolytic cleavage of KHG (51, 52). Those groups found to be essential for KHG-aldolase activity are also required for the enzyme to catalyze the decarboxylation of oxaloacetate. Figure 8 shows the effect of photooxidation in the presence of methylene blue on both of these enzymic activities of KHG-aldolase. At pH 8.0 and 23°C, 91% of the initial enzymic activity is lost within 10 minutes; both aldolase and β -decarboxylase activities are destroyed at the same rate and to the same extent.

The Effect of Substrates on the Rate of Photooxidation

In an effort to determine whether or not substrates had any protective effect on the rate or the extent of photoinactivation of KHG-aldolase, the following experiment was carried out: KHG-aldolase (1.50 to 2.00 mg), Tris·HCl buffer (100 μ moles, pH 8.0),

Legend to Figure 7

Glutathione (1.5 μ moles), buffer (100 μ moles), and methylene blue (160 nmoles) were mixed in a total volume of 1.0 ml. The solution was irradiated for 15 minutes at 23°C, as described in the Methods section. At appropriate times, 5 μ l aliquots were withdrawn and diluted into 0.1 M Tris·HCl buffer, pH 8.0, in spectrophotometer cuvettes. The sulfhydryl content of the solution was determined by adding 0.5 μ mole of DTNB and observing the absorbancy at 412 nm. Pseudo-first order rate constants were calculated from semi-logarithmic plots (percentage of initial sulfhydryl content versus time of irradiation). A control tube, identical in composition to the test solution, was kept in the dark for a 15 minute interval of time; it did not show any loss of sulfhydryl content during that interval of time. The following buffers were used at the pH values indicated: potassium phosphate, pH 6.0 to 7.5; Tris·HCl, pH 7.5 to 8.5. Variation in the rates of photooxidation observed between the two buffers was normalized by comparing the rate constants at pH 7.5 in both buffer systems.

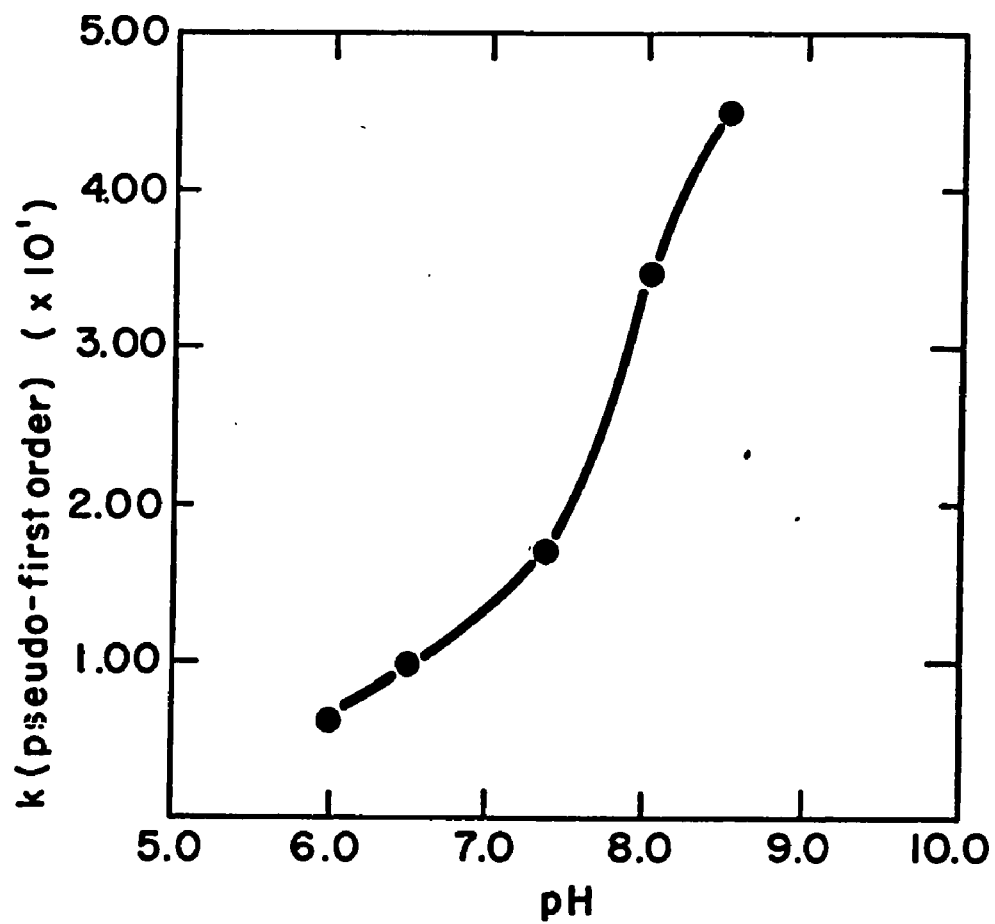


Figure 7. Photooxidation of the Sulfhydryl Group of Glutathione: pH-Rate Profile

Legend to Figure 8

KHG-aldolase (1.25 mg), Tris·HCl buffer (50 μ moles, pH 8.0), and methylene blue (40 nmoles) were mixed in a total volume of 0.5 ml. The solution was irradiated for 10 minutes, as described in the Methods section. At the times indicated, samples were withdrawn for determining the aldolase and the β -decarboxylase activities as follows: (a) KHG-aldolase activity; a 5 μ l aliquot was diluted (100-fold) into 0.05 M Tris·HCl buffer, pH 8.0, containing 5 mM 2-mercaptoethanol. The diluted samples were assayed for KHG-aldolase activity by the standard colorimetric procedure for glyoxylic acid. (b) β -Decarboxylase activity toward OAA; 10 μ l aliquots were removed at the times indicated; these samples were stored at 4°C in the dark until the end of the photooxidation process (10 minutes) at which time they were diluted into a β -decarboxylase assay system (see Methods section) and the decrease in absorbancy at 340 nm was followed. A control tube identical in composition to the test solution was stored in the dark; it showed no loss in either aldolase or β -decarboxylase activity during the 10 minute interval of time.

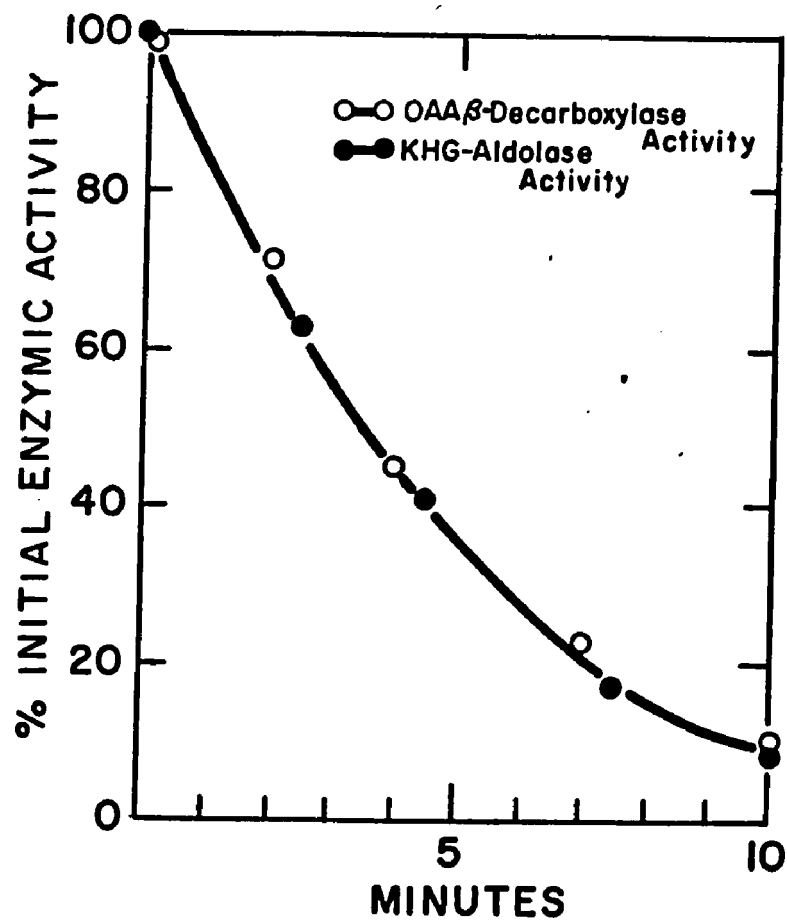


Figure 8. Effect of Photooxidation on the β -Decarboxylase and KHG-Aldolase Activities of the Enzyme

methylene blue (40 nmoles), and substrate (10 to 50 mM) were mixed in a final volume of 1.0 ml. The solution was exposed to the light source for 10 minutes. Aliquots (5 μ l) were withdrawn at appropriate times and diluted (100-fold) into 0.05 M Tris·HCl buffer, pH 8.0, containing 5 mM 2-mercaptoethanol. These diluted aliquots were assayed for KHG-aldolase activity by the standard colorimetric procedure for glyoxylic acid. In every case, the rate of inactivation was pseudo-first order; the percentage of initial enzymic activity remaining after a 10 minute exposure to light for enzyme photo-oxidized in the presence of various substrates is shown in Table 7. It can be seen that 50 mM pyruvate offers no protection against the process of photoinactivation. Glyoxylate, at a concentration of 50 mM, or a combination of glyoxylate plus pyruvate affords only 10 to 20% protection. Although 10 mM KHG provides only minimal protection, 30 mM concentrations of KHG protect up to 81% of the enzymic activity lost in the absence of any substrate. The analogs, α -ketobutyrate and α -ketoglutarate, were also tried in this respect but caused almost instantaneous bleaching of the methylene blue when irradiated. After the light was extinguished, the color of the dye slowly returned. The basis for this phenomenon is unknown.

Changes in Amino Acid Composition of KHG-aldolase Accompanying Photooxidation

KHG-aldolase, photooxidized by the usual procedure until 93% of its original enzymic activity was destroyed, was subjected to

Table 7

Effect of Substrates on the Photoinactivation
of KHG-aldolase

Substrate Added	% Initial Enzymic Activity (10 min, pH 8.0)	% Protection
None	3	--
50 mM Pyruvate	8	5
50 mM Glyoxylate	24	21
50 mM Pyruvate + 50 mM glyoxylate	16	13
10 mM KHG	25	22
30 mM KHG	84	81

amino acid analysis after acid hydrolysis. The amino acid composition of this photoinactivated enzyme is shown in Table 8 and compared with that of a sample of native KHG-aldolase. Of all the amino acids most commonly destroyed by photooxidation (histidine, methionine, tyrosine, cysteine, and tryptophan), only two appear to undergo significant alteration. These two aminoacyl residues are histidine and cysteine. Twenty-two out of a total of 24 histidyl residues and 8 out of 10 cysteinyl residues are destroyed in enzyme which has been 93% photoinactivated. Figure 9 is a pseudo-first order rate plot correlating the destruction of histidyl and cysteinyl residues in the protein molecule with the loss of aldolase activity. It can be seen that the rate constant for the destruction of histidyl residues is essentially equal to that for the inactivation of the aldolase, whereas the same constant for the loss of cysteinyl residues is only about one-half as large.

Substrate Stereospecificity of Photooxidized KHG-aldolase

It is not known what structural features of KHG-aldolase are responsible for its unusual ability to utilize either optical antipode of KHG as substrate. In order to ascertain whether the process of photooxidation might possibly have selectively destroyed enzymic activity toward a single isomer, photoinactivated KHG-aldolase was tested in this regard. The results are shown in Table 9. It can be seen that the enzyme simultaneously loses its ability to utilize the D- as well as the L-isomer of KHG.

Legend to Table 8

KHG-aldolase (2.3 mg), Tris·HCl buffer (100 μ moles, pH 8.0), and methylene blue (80 nmoles) were mixed in a total volume of 1.0 ml. This solution was exposed to light for 15 minutes. Aliquots (5 μ l) were removed at appropriate times, diluted (100-fold) into 0.05 M Tris·HCl buffer, pH 8.0, and stored in the dark for subsequent measurement of KHG-aldolase activity. After the light was extinguished, the photooxidation mixture was applied to a column of Sephadex G-25 (coarse, 0.9 x 13 cm) equilibrated with 0.05 M Tris·HCl buffer, pH 8.0, for purposes of removing the methylene blue. The column was washed with the same buffer solution and fractions of 0.5 ml were collected. The contents of the tubes containing protein were pooled and dialyzed against three one liter volumes of 1 mM NaCl solution adjusted to pH 7.0. This dialyzed solution was assayed for protein content, and then subjected to hydrolysis in 6 N HCl at 110°C for 24 hours. The hydrolysate was subsequently analyzed for the content of the individual amino acids.

Table 8

Amino Acid Composition of Native and
Photooxidized KHG-aldolase

Amino Acid	Residues per 120,000 gm	
	Native Enzyme	Photooxidized Enzyme
Lysine	39	40
Histidine	24	2
Arginine	56	54
Aspartate ^a	86	87
Threonine	63	64
Serine	76	77
Glutamate ^a	119	125
Proline	70	not measured
Glycine	116	112
Alanine	104	103
Valine	98	96
Methionine	9	9
Isoleucine	30	33
Leucine	134	139
Tyrosine	31	31
Phenylalanine	38	39
Cysteine ^b	10	2
Tryptophan ^c	15	15

^a Includes both the amide and free acid forms.

^b Determined by DTNB titration of the unhydrolyzed protein in 1% sodium dodecyl sulfate.

^c Determined spectrophotometrically.

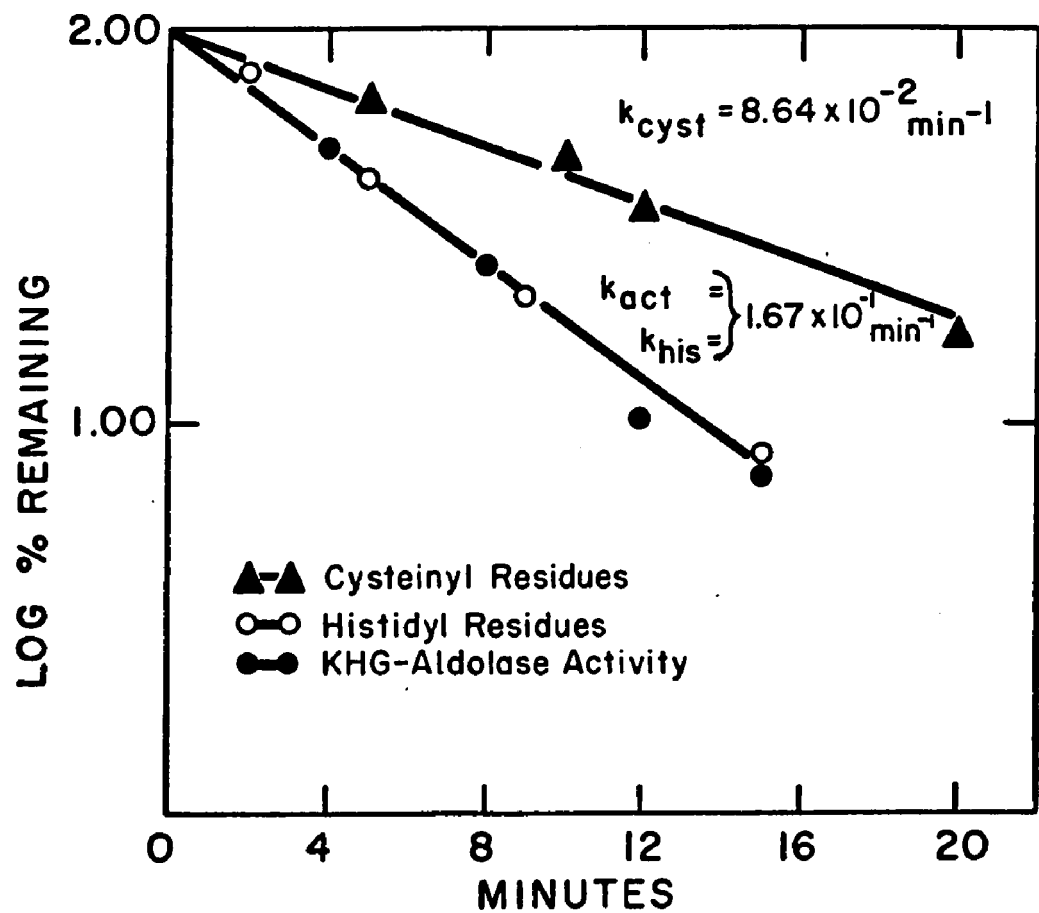


Figure9. Pseudo-first Order Rate Plot for Histidine and Cysteine Destruction During Photoinactivation of KHG-Aldolase

Table 9
Substrate Stereospecificity of
Photooxidized KHG-aldolase

Minutes of Photooxidation	% Initial Enzymic Activity Remaining	
	<u>D</u> -KHG	<u>L</u> -KHG
0	100	100
2	70	71
4	41	44
6	22	22
8	10	14
10	8	9

Reaction conditions were as described in Figure 8. Diluted aliquots were assayed for KHG-aldolase activity using both the D- and the L-isomer of KHG.

The Kinetic Constants of Photooxidized Aldolase

Table 10 lists the kinetic constants, determined by the method of Lineweaver and Burk (84), for native and for photo-oxidized KHG-aldolase. The K_m value does not change during the process of photoinactivation; in contrast, the value for V_{max} decreases in direct proportion to the loss of enzymic activity. Thus, the process of photooxidation is not simply producing a partially active enzyme with a lowered affinity for its substrate, but is totally destroying the capability of the aldolase to carry out catalysis (be that some part or all parts of the aldolytic reaction).

Schiff-base Formation by Photooxidized Aldolase

In order to determine whether or not photooxidized KHG-aldolase could still catalyze any part of the over-all reaction, the photoinactivated enzyme was tested for its ability to form Schiff-base intermediates with either pyruvate or glyoxylate. As seen in Table 11, enzyme which is subjected to all the photooxidation conditions, except exposure to light, still binds 0.81 mole of pyruvate and 1.05 moles of glyoxylate per mole (120,000 gm) of enzyme. In contrast, KHG-aldolase which is 55% photoinactivated loses an additional 41% of its initial enzymic activity after borohydride treatment in the presence of a substrate but is able to bind only 0.36 mole of pyruvate or 0.49 mole of glyoxylate per mole of aldolase. Aldolase which is 95% photoinactivated is unable to bind any radioactive substrate whatever in the presence of sodium borohydride. Thus,

Table 10
Kinetic Constants of Native and
Photooxidized KHG-aldolase

KHG-aldolase	K_m	V_{max}
	(molar)	(μ moles glyoxylate/20 min/mg protein)
Native	9.10×10^{-5}	100.0
Photooxidized (33% inactive)	8.35×10^{-5}	63.0
Photooxidized (91% inactive)	1.10×10^{-4}	7.2

Legend to Table 11

Two samples of KHG-aldolase (1.62 mg each) were photo-inactivated and then separated from the methylene blue, as described in Table 8. Photooxidation times for the two samples were 5 and 15 minutes, yielding enzyme preparations which had been inactivated 55% and 95%, respectively. Both samples, as well as native aldolase, were tested for their ability to form Schiff-base complexes in the following manner:

KHG-aldolase (222 μ gm), potassium phosphate buffer (50 μ moles, pH 6.0), and either ^{14} C-pyruvate (0.98 μ mole, 5.08 μ c/ μ mole) or ^{14} C-glyoxylate (1.01 μ moles, 4.12 μ c/ μ mole) were mixed in a total volume of 0.35 ml. This solution was treated alternately at 0°C with four 10 μ l portions of 1 M sodium borohydride and four 5 μ l portions of 2 M acetic acid over a period of 30 minutes. At the end of this time, the protein was precipitated with 230 mg of powdered ammonium sulfate and removed by centrifugation. The pellet was washed twice with 80% saturated ammonium sulfate solution and dissolved in 0.5 ml of 0.01 M potassium phosphate buffer, pH 7.4. This solution was then dialyzed exhaustively against the same buffer solution. The dialysate was assayed for KHG-aldolase activity, protein content, and level of radioactivity. From these data, the number of moles of radioactive substrate bound per mole (120,000 gm) of inactive KHG-aldolase was computed.

Table 11
Binding of ^{14}C -Labeled Substrates in the Presence of Borohydride by
Native and Photooxidized KHG-aldolase

Enzyme	Additions to Enzyme	Inactivation after NaBH_4 Reduction (%)	Radioactivity Bound (cpm/ μmole inactive protein)	^{14}C -Substrate Bound (moles/120,000 gm inactive protein)
Native control	^{14}C -Pyruvate + NaBH_4	95	5.96×10^6	0.81
Native control	^{14}C -Glyoxylate + NaBH_4	92	8.11×10^6	1.05
Photooxidized aldolase (55% inactive)	^{14}C -Pyruvate + NaBH_4	96	2.67×10^6	0.36
Photooxidized aldolase (55% inactive)	^{14}C -Glyoxylate + NaBH_4	96	3.79×10^6	0.49
Photooxidized aldolase (95% inactive)	^{14}C -Pyruvate + NaBH_4	98	0.26×10^6	0.04
Photooxidized aldolase (95% inactive)	^{14}C -Glyoxylate + NaBH_4	98	0.72×10^6	0.09

photoinactivated enzyme appears to be incapable of forming Schiff-base intermediates.

Ultraviolet Spectra of Native and Photooxidized KHG-aldolase

Because of the extensive destruction of histidyl and cysteinyl residues in the protein molecule by the process of photooxidation, it was of interest to see what differences might exist in the ultraviolet spectra of native and photooxidized enzyme. KHG-aldolase (2.3 mg) was photooxidized in the manner previously described at pH 8.0 in the presence of 80 μ M methylene blue; enzyme that was 95% inactivated was obtained. The methylene blue was removed using a column of Sephadex G-25, and the eluted protein was pooled and dialyzed against three 1 liter volumes of 0.02 M potassium phosphate buffer, pH 7.0. For a control, the aldolase was treated in exactly the same manner except it was not exposed to light. The ultraviolet spectra of these samples of KHG-aldolase are shown in Figure 10. A small increase in absorbancy can be seen for the photooxidized protein in the region from 300 to 330 nm; however, the main difference lies in the portion of the spectrum between 245 and 265 nm. The maximum difference occurs at 253 nm; the photooxidized sample absorbs 0.17 optical density units more than the native enzyme at this wavelength.

Total Cysteine-cystine Content of KHG-aldolase

The only information concerning the number of sulfhydryl groups in the KHG-aldolase molecule comes from DTNB-titrations

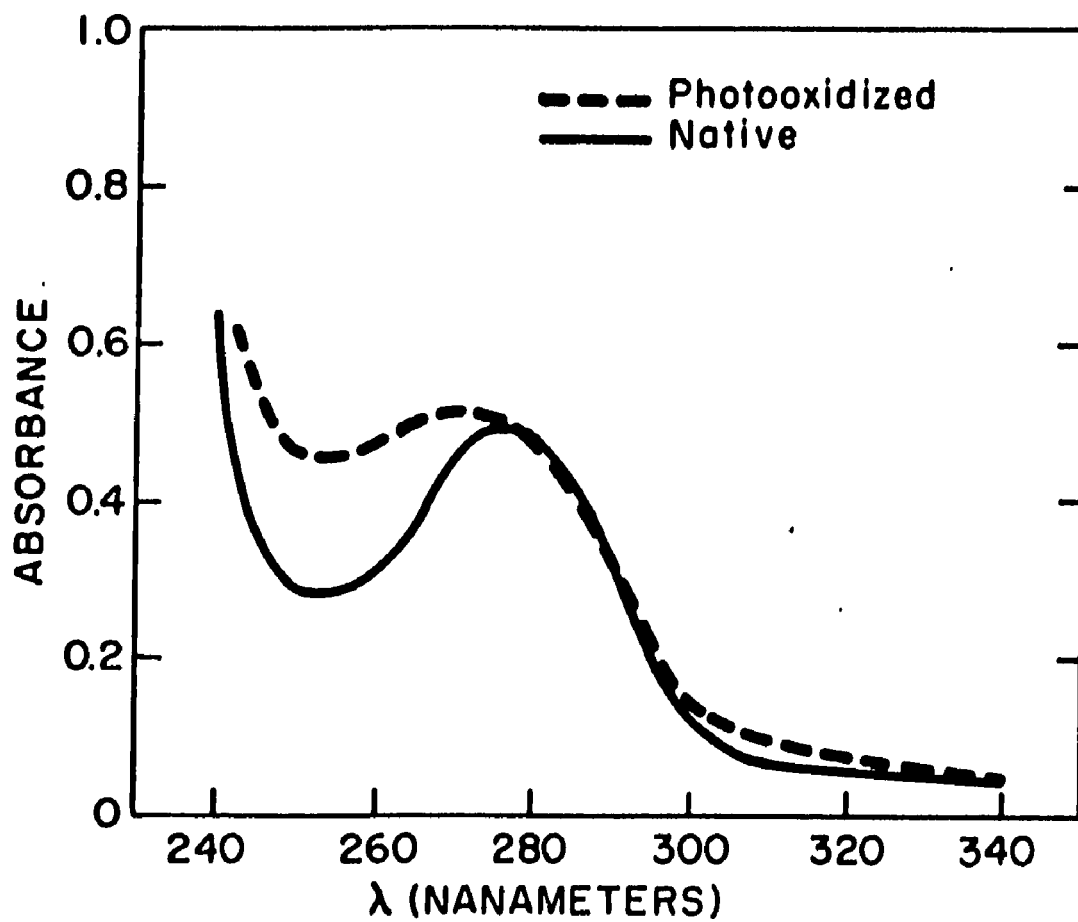


Figure 10. Ultraviolet Spectra of Native and Photooxidized KHG-Aldolase

of the enzyme in the presence of denaturants (51). By titration with DTNB, Lane and Dekker found a total of 8 sulfhydryl groups in aldolase which had been denatured in 1% sodium dodecyl sulfate or 6 M guanidine hydrochloride. Recently, in some of my work with preparations of the aldolase showing only one protein band in polyacrylamide gel electrophoresis, it has been possible to titrate a total of 10 sulfhydryl groups with DTNB in 1% SDS. Because of this apparent variability in the sulfhydryl content of KHG-aldolase preparations, it was decided to examine the titration of the sulfhydryl groups of the enzyme with another reagent, and also to determine the total cysteine and cystine content of the aldolase.

Titration of the Sulfhydryl Groups of KHG-aldolase with
p-Hydroxymercuribenzoate (PMB)

A typical titration curve of KHG-aldolase using PMB is shown in Figure 11. A small secondary increase in the absorbance at 250 nm can be seen after the equivalence point is reached. This secondary increase in absorbance is not unusual and has been attributed to the reaction of PMB with other groups in protein molecules (76). The equivalence point in Figure 11 occurs at 0.0330 ml of PMB added which, by calculation, corresponds to the titration of 10.15 sulfhydryl groups per aldolase molecule. This same titration carried out in 1% SDS resulted in the reaction of 10.00 sulfhydryl groups per KHG-aldolase molecule. For this

Legend to Figure 11

KHG-aldolase (0.447 mg), and Tris·HCl buffer (10 μ moles, pH 7.4) were mixed in a final volume of 1.0 ml. The titration was performed at 25°C by adding 5 μ l aliquots of 1.143 mM PMB and following the increase in absorbancy at 250 nm. A blank containing no enzyme was also titrated; any absorbance observed was subtracted from that obtained for the protein reaction mixture. Small corrections were also made for volume increases.

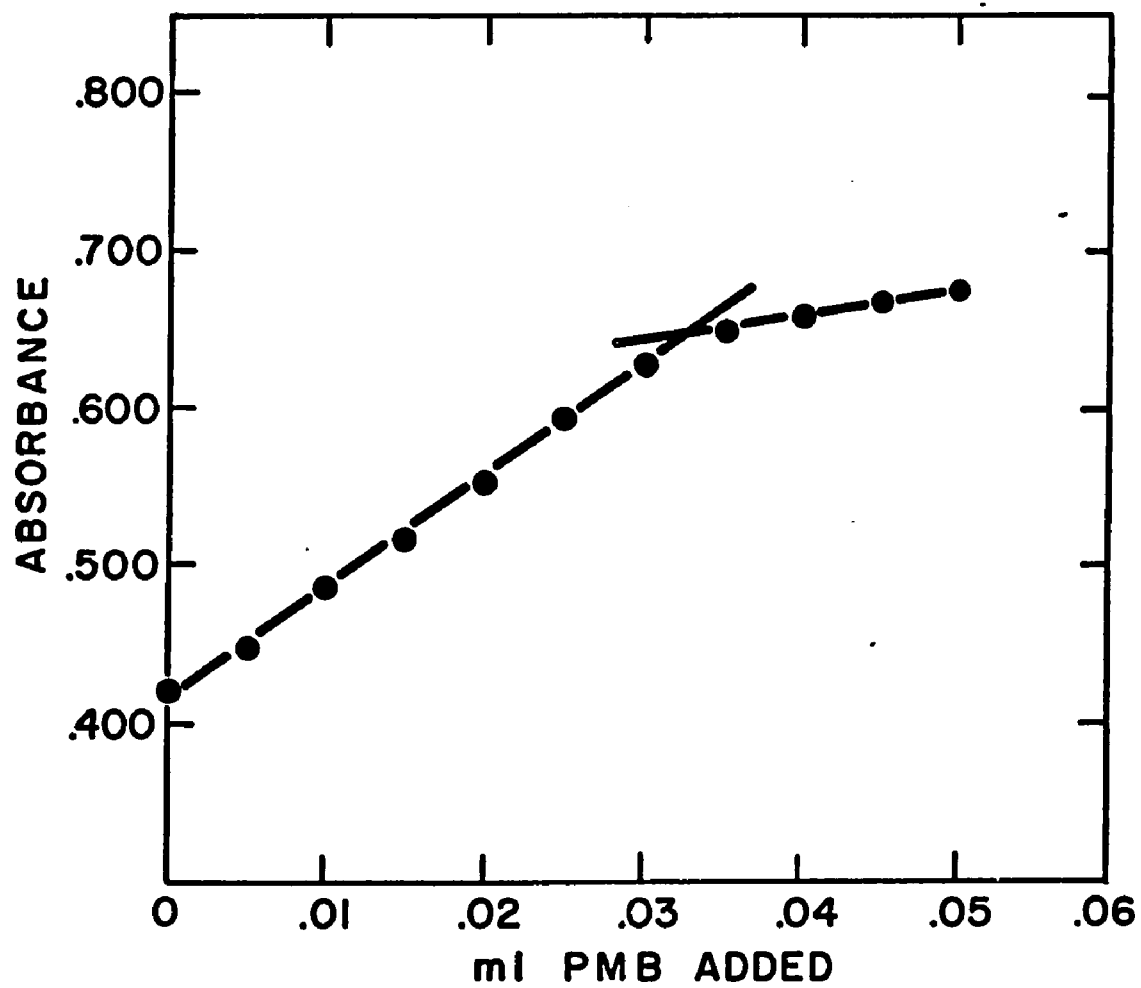


Figure II. PMB Titration of KHG-Aldolase

enzyme preparation, a total of 9.78 sulfhydryl groups could also be titrated by DTNB in 1% SDS.

Protection of Sulfhydryl Groups by Substrates Against PMB Titration

The presence of a substrate, pyruvate or glyoxylate, during the titration of KHG-aldolase with DTNB in the absence of a denaturant resulted in the protection of 1 sulfhydryl group out of 4 titratable with no substrate added (51). This finding suggested that a cysteinyl residue might be located at the active site of the enzyme. Because apparently more sulfhydryl groups could be titrated in the KHG-aldolase molecule with PMB in the absence of denaturants, we wondered whether any such groups were also protected by substrates during PMB-titration and, if so, how many residues were involved. The titrations were carried out as described in the previous section, with the addition of substrate where indicated. Table 12 shows the results of titrating KHG-aldolase with PMB in the presence and absence of substrates. It can be seen that in this case also glyoxylate protects about 1 sulfhydryl group and pyruvate about 1 to 2 from reacting with PMB. Similar experiments with other preparations of KHG-aldolase showed that either substrate protected 1 to 2 sulfhydryl groups against titration by PMB. The same degree of protection was seen in the presence of 50 mM glyoxylate as was observed with a concentration of 10 mM. Because it absorbs strongly at 250 nm, pyruvate could not be tested at concentrations above 10 mM.

Table 12

Titration of KHG-aldolase with PMB in the
Presence and Absence of Substrates

Addition to Enzyme	Number of Sulfhydryl Groups Titrated	Number of Sulfhydryl Groups Protected
None	10.30	----
1% SDS	10.00	----
10 mM Glyoxylate	9.31	0.99
10 mM Pyruvate	8.51	1.79

Inactivation of KHG-aldolase Activity During Reaction with PMB

Table 13 shows the loss of catalytic activity incurred by KHG-aldolase when the sulfhydryl groups in the molecule are titrated by PMB. The aldolase preparation used in these experiments was found to have only eight reactive sulfhydryl groups. At least 2 of these groups may be titrated without essentially any loss of enzymic activity. After 4 to 5 sulfhydryl groups are titrated, about 50% of the catalytic activity has been destroyed. The final 3 groups are titrated with a further decrease in aldolase activity to a level about 15% of that observed with the native enzyme. If KHG-aldolase titrated to this extent (i. e. 85% inactivated) is allowed to stand in solution for some time (approximately 1 to 2 hours), turbidity develops in the solution and a precipitate finally forms. This precipitation might result from a disruption of the native conformation of the enzyme yielding a less soluble form of the protein. If the PMB-titrated aldolase (85% inactivated) is exposed to 0.1 M 2-mercaptoethanol soon after the titration is completed, 70 to 80% of the initial enzymic activity may be restored. Apparently KHG-aldolase does not undergo much irreversible conformational change if treated in this manner.

Total Cysteine-cystine Content of KHG-aldolase

A portion of a KHG-aldolase preparation determined to be homogeneous on polyacrylamide disc gel electrophoresis was dialyzed against three 1 liter volumes of 0.01 M NaCl for 24 hours. The

Legend to Table 13

KHG-aldolase (0.935 mg), and Tris·HCl buffer (100 μ moles, pH 8.0) were mixed in a total volume of 1.0 ml at 26°C. PMB (0.544 mM) was added in 5 μ l aliquots and the absorbancy change at 250 nm was measured. After each 5 μ l addition, an aliquot (2 μ l or 5 μ l) was withdrawn from the titration mixture and diluted into 1.0 ml of 0.1 M Tris·HCl buffer, pH 8.4, containing 5 μ moles of KHG at 37°C. After the reaction mixture was incubated for 10 minutes at 37°C, 0.4 ml of 12% metaphosphoric acid solution was added and the quantity of glyoxylate in the assay mixture was determined by the usual colorimetric assay. The percentage of initial enzymic activity remaining and the sulfhydryl titer were calculated after each addition of PMB.

Table 13

Inactivation of KHG-aldolase Activity During

Titration by PMB

Number of Sulfhydryl Groups Titrated	% Initial Enzymic Activity Remaining
0	100
0.85	101
1.44	101
2.88	95
4.55	58
5.00	43
7.98	15

resulting solution was assayed for protein content and found to contain 0.966 mg/ml of protein. One and one-half ml of this solution was pipeted into a constricted tube along with 1.5 ml of constant boiling HCl. To this solution was added 0.06 ml reagent grade dimethylsulfoxide. The resulting solution was prepared for hydrolysis as described in the Methods section. Hydrolysis was carried out at 110°C for 22 hours; the hydrolysate was prepared for amino acid analysis as also described in the Methods section. The final residue was dissolved in 1 ml of 0.02 M sodium citrate buffer, pH 2.2. Cysteic acid content was calculated as described by Spencer and Wold (79) using the constants for aspartic acid and for alanine. In either case the 1/2 cystine content of KHG-aldolase was found to be 14 residues per mole (120,000 gm) of aldolase.

DISCUSSION

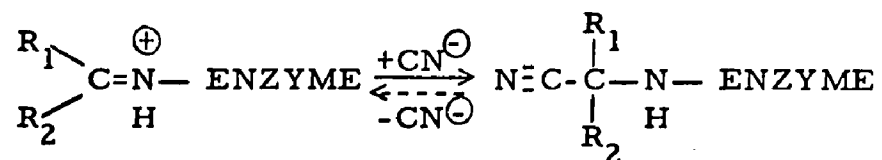
Aldolases have been divided into two classes based on their chemical and enzymic properties (16). Class I aldolases, the prototype of which is FDP-aldolase isolated from rabbit muscle, contain no metal ions bound to the protein. Their catalytic activity is not enhanced by the addition of mono- or divalent metal ions, nor is it inhibited by chelating agents such as EDTA. As a group, Class I aldolases catalyze aldol cleavage or addition reactions by a mechanism involving the formation of a Schiff-base intermediate between the ϵ -amino group of one or more lysyl residues in the enzyme and the carbonyl group of a substrate which is capable of undergoing keto-enol tautomerization. Class II aldolases, the prototype of which is yeast FDP-aldolase, are metalloenzymes. The metal ion is postulated as being at the active site of the enzyme, since Class II aldolases are subject to competitive inhibition by EDTA. Aldolases of this class are activated by $K^{\oplus}$ and other mono- and divalent metal ions. To date, there is no evidence for Schiff-base intermediates being involved in reactions catalyzed by Class II aldolases. Workers in the field believe that the metal ion may act to enhance enolization of the carbonyl substrate thereby eliminating the need for any Schiff-base intermediate.

KHG-aldolase, isolated from bovine liver extracts, possesses several chemical and enzymic properties which place it among the Class I aldolases. It does, however, have several unique and

unexpected properties not characteristic of other Class I aldolases such as FDP-aldolase, KDPG-aldolase, and transaldolase. These latter aldolases form Schiff-base intermediates with only those substrates which one would predict on a mechanistic basis. These substrates are FDP and DHAP with FDP-aldolase, KDPG and pyruvate with KDPG-aldolase, and DHAP with transaldolase. None of these aldolases forms a Schiff-base intermediate with its third substrate, glyceraldehyde-3-phosphate. KHG-aldolase, in contrast, is capable of forming a Schiff-base intermediate with any of its substrates (i. e. KHG, pyruvate, glyoxylate, or formaldehyde). KHG-aldolase also is unusual in that it is a very effective β -decarboxylase; the bovine liver enzyme catalyzes the decarboxylation of OAA at about 50% the rate of KHG cleavage. A third unique property of KHG-aldolase is its ability to catalyze the aldol cleavage or formation of either optical antipode of KHG. Both isomers of this substrate are utilized at approximately the same rate and to the same extent.

Yet another peculiar property of KHG-aldolase is reported in this thesis. This aldolase is irreversibly inactivated by cyanide in the presence of glyoxylate, formaldehyde, or certain carbonyl analogs of these compounds. Other Class I aldolases have been reported to show substrate-dependent inhibition by cyanide (40, 41) in the presence of an appropriate substrate, but in each case the observed inhibition was reversed by dilution of the cyanide

concentration. Based on chemical precedent (60), this reversible inhibition of other Class I aldolases was postulated as being due to the addition of cyanide to a Schiff-base intermediate resulting in the formation of an enzyme-bound aminonitrile, as shown below:



The formation of such an enzyme-bound aminonitrile has now been verified experimentally for the first time. The radioactive product resulting from acid hydrolysis of KHG-aldolase, previously inactivated by cyanide in the presence of ^{14}C -formaldehyde, has been isolated and identified. When this radioactive product was examined by paper chromatographic techniques, it migrated identically with the expected product, N^6 -carboxymethyllysine, in 5 different solvent systems (see Table 4). The mole per mole stoichiometry of the radioactive adduct was found to be 1:1:1 (formaldehyde: cyanide:KHG-aldolase) as would be expected on the basis of aminonitrile formation (Table 3).

Examination of the kinetics of this cyanide inactivation of KHG-aldolase revealed that it is a biphasic process. Biphasic inactivation kinetics of this nature had been observed earlier by Brand and Horecker (41) in studies concerning the reversible inhibition of transaldolase by cyanide in the presence of fructose-6-phosphate (F-6-P). Transaldolase forms a very stable Schiff-base

intermediate with DHAP; this intermediate can be separated from unreacted F-6-P and reaction products on a column of Sephadex G-25. If they treated this isolated intermediate with cyanide, only the rapid phase of the biphasic inhibitory process was observed. These authors, therefore, proposed that two competing reactions occurred during the reversible inhibition of transaldolase by cyanide: (1) the addition of cyanide to the Schiff-base intermediate resulting in inhibition, and (2) cyanohydrin formation between cyanide and the carbonyl substrate. They attributed the rapid phase of this inhibition process (observed with either the isolated transaldolase-DHAP intermediate, or when transaldolase was treated directly in the presence of F-6-P with cyanide) to the fast addition of cyanide to the Schiff-base intermediate. The slow portion of the biphasic inhibition curve (observed only with transaldolase treated directly with cyanide in the presence of F-6-P) was then due to the "...continued formation of complex (Schiff-base intermediate) between free enzyme and F-6-P which is freed from cyanohydrin adduct."

This type of competing reaction scheme has been considered for the irreversible inactivation of KHG-aldolase by cyanide in the presence of glyoxylate or its analogs. Initial experiments indicated that in the case of KHG-aldolase, however, a third reaction had to be added to account for the observed kinetics. This third competing equilibrium is a direct interaction of cyanide with the free aldolase,

which allows for a more extensive rapid rate of inactivation once the substrate is added (Figure 3). The overall set of reactions involved in this case is outlined in Figure 12. Experiments in which the time of preincubation (Figure 4) and those in which the concentrations of carbonyl substrate (Figure 5) were varied are, for the most part, consistent with this scheme; one discrepancy, however, appears to exist. The sharp break in the kinetic curves (Figure 3) can be explained on the basis of rapid cyanohydrin formation (complete within 1 minute) and a subsequent slow dissociation of that adduct (accounting for the slow phase of the inactivation process). This would mean that within 1 minute after mixing cyanide and substrate, a new steady-state concentration of carbonyl substrate would be attained. The data in Figure 4-b, however, have been interpreted as indicating a rate of cyanohydrin formation slow enough to still be measurable after 10 to 15 minutes.

It may be that a modification of the scheme shown in Figure 12 would satisfy the data, or that a new interpretation is needed for part or all of the reactions listed. More detailed experimentation using rapid reaction techniques might be helpful in examining the first, fast phase of this inactivation process. In view of the many and varied effects cyanide is known to have on enzyme-catalyzed processes, it is possible that other reactions of cyanide with free aldolase should be added to the scheme shown in Figure 12 in addition to the one already included. The nature of the direct interaction of

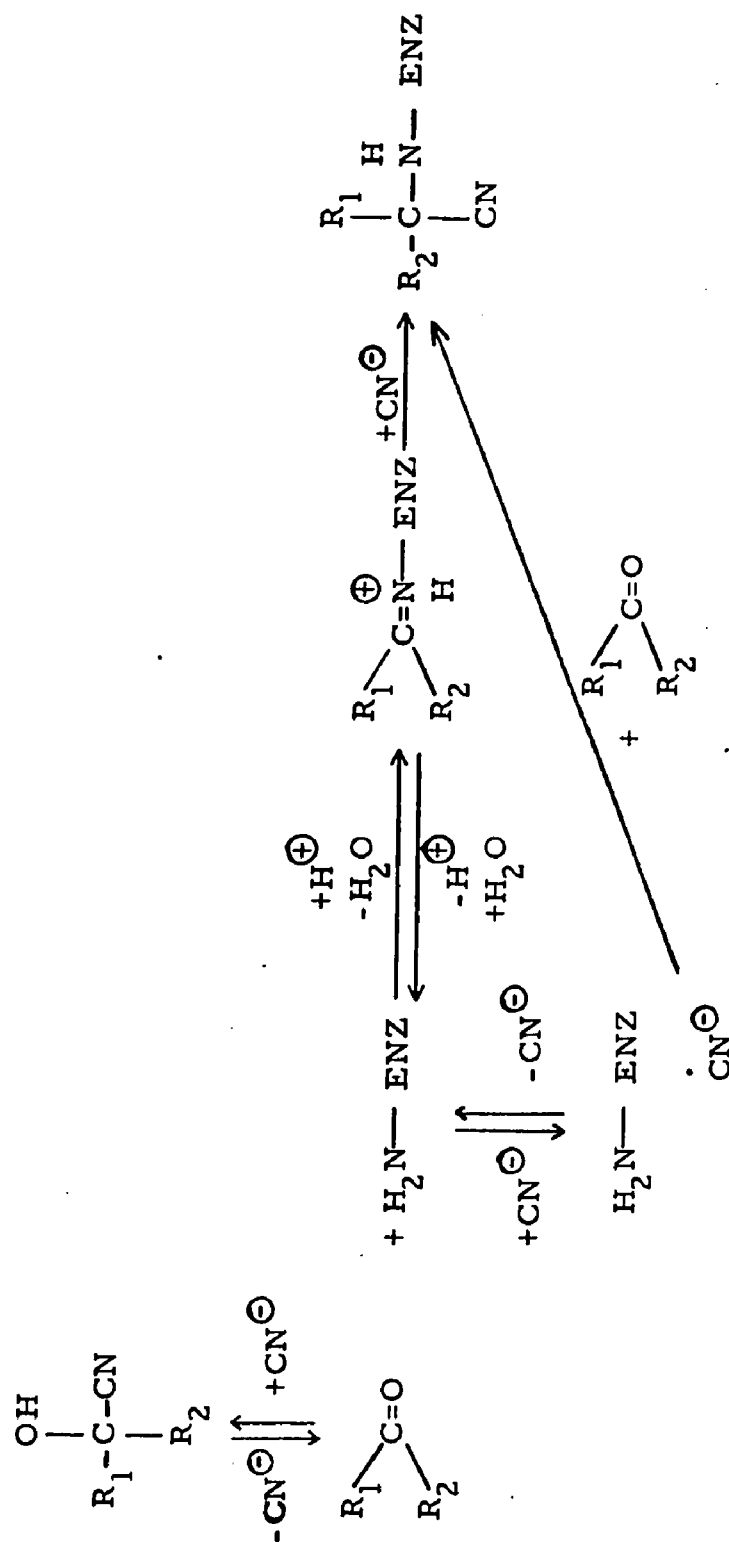


Figure 12. Proposed Scheme of Competing Reactions Occurring During the Inactivation of KHG-aldolase by Cyanide in the Presence of a Carbonyl Substrate

cyanide with KHG-aldolase has been the subject of some experiments described in this thesis. In order to account for the observed kinetics, it seems most likely that this interaction is a freely reversible "binding" which occurs at or near the active site, resulting in an increased effective concentration of cyanide in the region of Schiff-base formation. Because of the measurable effect varying intervals of time have on this "interaction" (Figure 4-c), something other than a strictly ionic attraction seems to be indicated. Some of the known reactions of cyanide can be discounted on the basis of facts already known about KHG-aldolase. For instance, complexation by cyanide of a metal ion does not seem applicable since the aldolase does not appear to contain any metal, nor is a metal ion apparently required for enzymic activity. In addition, KHG-aldolase has no essential carbonyl residue in the molecule (such as pyruvate or α -ketobutyrate), since no loss of enzymic activity is observed after it is treated with borohydride in the absence of a substrate (47). Of course, the possibility of cyanohydrin formation with a nonessential carbonyl moiety in the protein molecule is not precluded but, again, it must be noted that no evidence exists indicating that KHG-aldolase contains such a residue. Cleavage of a disulfide bond in the enzyme molecule by reaction with cyanide also does not appear likely for two reasons. Disulfide bond scission is essentially irreversible (as shown in the Results section) and therefore would not account for the observed kinetics. Secondly, experiments in which the number of

sulfhydryl groups present in KHG-aldolase was determined before and after exposure to cyanide (Table 5) indicate no increase in the number of titratable sulfhydryl groups after treatment with cyanide as would be expected in the event of disulfide bond cleavage. One must remember, in addition, that incubation of the aldolase with cyanide in the absence of a carbonyl substrate followed by exhaustive dialysis results in no loss of enzymic activity (Table 3).

Although not absolutely impossible, it is difficult to envision the cleavage of one or two disulfide bonds in a protein occurring with complete retention of its enzymic activity. The CD spectrum of KHG-aldolase in the presence of cyanide indicates that no gross conformational changes occur; such results are also highly unlikely in the event of disulfide bond scission.

Although KHG-aldolase is not inactivated by cyanide in the absence of a carbonyl substrate, the enzyme does "bind" about 0.4 mole of ^{14}C -cyanide per mole of protein (Table 6) when only aldolase and radioactive cyanide are incubated together. This "bound" ^{14}C -cyanide can be partially but not completely removed by dialyzing the protein solution exhaustively against a solution of 0.5 M KCl. Some portion of the "bound" ^{14}C -cyanide, therefore, appears to be "held" by ionic interactions, possibly at a site (or sites) on the enzyme which normally binds the anionic carboxylate groups of the substrate, KHG. About 0.09 mole of ^{14}C -cyanide (or approximately 1/4 of the total amount "bound") routinely remains attached to the

enzyme after dialysis against 0.5 M KCl solution; this amount of cyanide, therefore, would appear to be "bound" in a manner different than the other 75%. Such so-called "tightly-bound" ^{14}C -cyanide seems not to be held in a readily reversible manner, however, since it is not exchangeable with unlabeled cyanide (Table 6).

The following hypothesis is presented as a reasonable explanation for the proposed interaction of cyanide with free enzyme as depicted in Figure 12. Cyanide induces a small conformational change in the protein (an indication of such a change is observed in the CD spectrum for KHG-aldolase in the presence of cyanide). This change in conformation enables the cyanide to penetrate a region of the active site of the aldolase which was previously inaccessible and to which the cyanide can now "bind" electrostatically. The rate of this conformational change can then account for the anomalously slow rate of the apparent ionic interaction between enzyme and cyanide.

The role of an active-site lysyl residue(s) in KHG-aldolase catalysis has been well established (47). Studies in which the enzyme was treated with sodium borohydride in the presence of an appropriate substrate have shown unequivocally that a lysyl residue participates in the formation of Schiff-base intermediates. Results presented in this thesis concerning cyanide inactivation of KHG-aldolase activity substantiate the involvement of a lysyl residue(s) in the catalytic process. In an effort to determine what other aminoacyl

residues might possibly be involved in KHG-aldolase catalysis, photooxidation studies were carried out.

Photooxidation of KHG-aldolase in the presence of methylene blue results in a rapid loss of enzymic activity; the rate of this process is pseudo-first order. The photooxidized aldolase is inactive with both the D- and the L-isomers of KHG. In addition, its β -decarboxylase activity toward OAA is destroyed. All of these enzymic activities of KHG-aldolase are lost at the same rate and to the same extent, lending further support to the hypothesis that a common residue(s) in the enzyme is involved in both aldolase and β -decarboxylase activities.

The loss of KHG-aldolase activity during photooxidation is paralleled by extensive destruction of histidyl and cysteinyl residues in the enzyme. Amino acid analyses show a loss of 22 out of 24 total histidyl residues; 8 out of 10 cysteinyl residues are also destroyed. Data obtained from amino acid analyses on 5 samples of KHG-aldolase photoinactivated 0, 17, 50, 78, and 98 percent were analyzed according to the method of Ray and Koshland (85). The apparent first-order rate constants for the loss of activity and for the destruction of histidyl residues were found to be identical ($1.67 \times 10^{-1} \text{ min}^{-1}$). The corresponding rate constant for the destruction of cysteinyl residues was only one-half as large ($8.64 \times 10^{-2} \text{ min}^{-1}$). These data can be interpreted as indicating that the loss of enzymic activity is due either to the destruction of one essential histidyl residue or

one of two essential cysteinyl residues. This interpretation must be viewed with extreme caution, however, since it is based on the assumption that no secondary effects, such as disruption of protein conformation, have occurred during the course of the photooxidation process. An assumption such as this could well be unwarranted when extensive modification of aminoacyl residues occurs concomitant with loss of enzymic activity. Thus, although either cysteinyl or histidyl residues (or both) are essential for the enzymic activity of KHG-aldolase, it would be presumptuous to estimate the number of each residue involved on the basis of the experimental data so far obtained.

The pH-rate profile for the photoinactivation of KHG-aldolase is consistent with that found for the photooxidation of free histidine in solution as well as for that obtained for the photooxidation of the sulfhydryl group of glutathione (see Results section). The apparent pK for the overall photoinactivation process is about 7.3. This value is well below what one would expect for the pK_a of the sulfhydryl group of normal cysteinyl residues in proteins; such sulfhydryl groups have pK_a values between 8.5 and 9.2. The pK obtained from the pH-rate profile for the photooxidation of the sulfhydryl group of glutathione is also below its normal value. In this case, the rate determining step in the process may involve a species with a lowered pK_a value due to interaction with the dye. Lane (51) noted earlier, with regard to the pH-rate profile of the

tetranitromethane-dependent oxidation of sulfhydryl groups in KHG-aldolase, that the pK of this process was 7.4 to 7.5, a value also anomalously low for cysteinyl sulfhydryl groups. With this reagent, however, no pH dependency was observed for the oxidation of the sulfhydryl group of glutathione. Lane proposed that a conformational change possibly occurred in the enzyme at pH 7.4, which exposed the sulfhydryl groups of KHG-aldolase to TNM and accounted for the pH dependency of the reaction. Such an explanation of the pH-rate profile for photooxidation of KHG-aldolase would be an alternative consistent with the destruction of essential cysteinyl residues in the molecule. The pH-rate profile obtained for the photoinactivation of KHG-aldolase is the same as that reported by other authors for the photodestruction of either free histidine or of histidyl residues in certain enzymes (61, 63, 83).

In an attempt to selectively modify only histidyl residues in the KHG-aldolase molecule by photooxidation, the enzyme was exposed to a concentration of PMB sufficient to react with all the sulfhydryl groups prior to subjecting the protein to photooxidation. This method of protecting sulfhydryl groups of proteins during the process of photooxidation has been used successfully with 6-phosphogluconate dehydrogenase (83), and also in our laboratory for protecting the sulfhydryl group of bovine plasma albumin against photooxidation in the presence of methylene blue. Surprisingly, the cysteinyl residues of KHG-aldolase exposed to an excess of PMB

were still photooxidized at the same rate and to the same extent as those in the native enzyme.

Comparison of the ultraviolet spectra of native and photo-oxidized KHG-aldolase shows a major increase in absorbance in the 245 to 265 nm region. A similar observation has been made before, using either lactate dehydrogenase (86) or 6-phosphogluconate dehydrogenase (83) when these enzymes were individually photo-oxidized in the presence of methylene blue. In the former case, the authors indicated that the observed rise in absorbance could be attributed to the presence of ultraviolet-absorbing materials extracted from the charcoal which they used to remove the methylene blue. In the latter case, however, charcoal treatment was not used to remove the methylene blue; instead, chromatography on a column of Sephadex G-25 was utilized for this purpose. In the latter instance, therefore, it was proposed that the observed increase in absorbance was due to the formation of oxidation products of histidine, and one such product with maximal absorbance at 244 nm was suggested to be acetylimidazole. Since that report appeared in the literature, another paper which characterized the products of the dye-sensitized photooxidation of free histidine and N-benzoylhistidine was published (87). These photooxidation processes were carried out using either riboflavin, rose bengal, or methylene blue as the photosensitizer; the same products were obtained in each case. It was determined that acetylimidazole was not among the products, nor

did any of the compounds which were characterized have a maximum absorbance above 230 nm. One product did have a secondary maximum ($\epsilon_M = 7,000$) at 275 nm. It must be kept in mind, however, that the products resulting from photooxidation of free histidine may not necessarily be identical to those formed during the photooxidation of histidyl residues in a protein. In addition, the wavelength for maximal absorbance of protein-bound products may differ from that determined for the same compounds free in solution.

Photooxidation of KHG-aldolase results in no significant change in the K_m of the enzyme for KHG. Rather, the entire effect of photooxidation is reflected in a lowered value for V_{max} . Thus, the enzyme is catalytically inactive and not simply insensitive to the concentration of substrate being employed. The photoinactivated enzyme has apparently lost the ability to form Schiff-base intermediates with substrates (Table 11), even though the active-site lysyl residue(s) has not been destroyed by this treatment. A catalytic step occurring before Schiff-base formation may possibly be blocked by photooxidation. As has been indicated earlier in the Introduction, results have been obtained using FDP-aldolase which suggest that there is an initial recognition and binding of the substrate, FDP, prior to actual Schiff-base formation. This step is thought to involve the binding of phosphate moieties in the substrate to positively charged groups in the protein molecule. It seems reasonable to postulate a similar initial "recognition and binding"

step in the reaction catalyzed by KHG-aldolase. In this case, the initial recognition would involve not phosphate anions but carboxylate groups in the substrate. These groups would be attracted to positively charged clusters in or near the active site of the aldolase. Histidyl residues in KHG-aldolase would be capable of existing as positive ions and these same residues have been observed to be substantially destroyed during the process of photooxidation.

Another possible explanation for the failure of photoinactivated KHG-aldolase to form Schiff-base intermediates is that a disruption of the conformation of its active site has occurred. The pK_a value of the active-site lysyl residue is believed to be unusually low for an ϵ -amino group of a lysyl residue (usual $pK_a \approx 10$). This abnormally low pK_a value accounts for its ability to function in Schiff-base formation at pH values of 7 to 8, as it is the unprotonated species that participates in the reaction. This lysyl residue, therefore, is probably in a highly hydrophobic active-site environment which protects it against protonation by solvent molecules. Such factors have been shown to be true for the active site in FDP-aldolase (44). If the process of photooxidation results in the opening up of the active site so that it is now exposed to solvent molecules, the pK_a of this unique lysyl residue would rise and approach its more normal value of about 10. Such a change would effectively prevent it from participating in Schiff-base formation. Quite obviously, a conformational change allowing for such phenomena could result from the

destruction of either histidyl or cysteinyl residues. In a report concerning the oxidation of sulfhydryl groups in KHG-aldolase by TNM, Lane (51) described the effects of this process on the catalytic activity of the enzyme. After 4 sulfhydryl groups were oxidized, it was observed that this kind of modified aldolase was also unable to form Schiff-base intermediates with either pyruvate or glyoxylate.

One other case in which photooxidation of an enzyme resulted in an inability to form Schiff-base intermediates with substrates, even though the active site lysyl residue remained unchanged, is that of transaldolase isolated from Candida utilis (65). Complete enzymic inactivation occurred concomitant with the destruction of one out of two histidyl residues in the enzyme when photooxidation was carried out in the presence of rose bengal. No other aminoacyl residue was reported to be lost during this process (however, no complete amino acid analysis before and after photooxidation was reported). In this case, the inactivation process was attributed to a destruction of that group in the enzyme (namely, the imidazolium group of histidine) responsible for abstracting a proton from the substrate (i. e. , F-6-P). The failure of the photooxidized enzyme to form a Schiff-base intermediate is probably best understood after one considers the unusual mechanism of action of transaldolase. No Schiff-base intermediate between F-6-P and transaldolase has ever been detected by the method of borohydride reduction; only

the DHAP-transaldolase Schiff-base intermediate has been successfully reduced and identified by this technique (39,42). This result has led to the proposal of concerted, rather than stepwise, Schiff-base formation and aldol cleavage for this enzyme. If these two steps are concerted, then Schiff-base formation cannot occur if the cleavage reaction is obstructed. Since the abstraction of a proton from the substrate is an integral part of the cleavage reaction, photooxidized transaldolase is, therefore, unable to catalyze any part of the aldol reaction. An explanation of this nature does not seem to be relevant to the failure of photooxidized KHG-aldolase to form Schiff-base intermediates with pyruvate or glyoxylate, since in this case the aldol reaction most likely occurs by a stepwise mechanism similar to that of FDP- and KDPG-aldolases. KHG-aldolase, like the two enzymes just mentioned, stably binds both substrates one would expect on a mechanistic basis when it is treated with these substrates in the presence of sodium borohydride (these substrates are KHG and pyruvate in the KHG-aldolase reaction). In addition, KHG-aldolase can form a Schiff-base intermediate with its third substrate, namely, glyoxylate (KHG cleavage) or formaldehyde (KHB cleavage). This binding of glyoxylate or formaldehyde lends additional support to a stepwise mechanism for KHG-aldolase since even the abstraction of a proton cannot occur with the binding of these two substrates.

The cysteinyl residues of KHG-aldolase have been modified by several techniques. These residues display varying reactivities toward the different reagents employed. TNM and DTNB react with only a few sulfhydryl groups in the native enzyme, while photooxidation in the presence of methylene blue or PMB titration results in nearly complete modification of all the cysteinyl residues.

PMB reacts rapidly with KHG-aldolase, titrating a total of 10 thiol groups in the native enzyme. An identical number of these groups react with PMB when the titration is performed in 1% SDS. These results are in sharp contrast with those obtained using DTNB to titrate the sulfhydryl groups in KHG-aldolase (51). With this reagent, only four of these groups react in the native enzyme even after 120 minutes. If this titration is carried out in 1% SDS, DTNB is found to react with a total of 8 to 10 thiol groups.

Either 10 mM pyruvate or 10 mM glyoxylate appears to protect 1 to 2 cysteinyl residues in the native enzyme from reaction with PMB (Table 12). The same observation has been made during the titration of native KHG-aldolase with DTNB in the presence of either 5 mM glyoxylate or 5 mM pyruvate; one sulfhydryl group was protected from reaction with DTNB by either substrate. When these results were obtained with DTNB, it was proposed that the observed protection was due to a substrate-induced change in conformation of the protein molecule which made one cysteinyl residue inaccessible to DTNB, rather than as a result of a true "masking" of the thiol

function by the substrate. The explanation proffered was supported by data indicating that either pyruvate or glyoxylate provided a protective effect against the unfolding of KHG-aldolase in 8 M urea. This hypothesis seems adequate to account for the same kind of protection one sees when the enzyme is reacted with PMB in the presence of either pyruvate or glyoxylate. An interesting experiment, which also lends support to the theory of a substrate-induced change in conformation, is the one in which the number of sulfhydryl groups in KHG-aldolase was determined before and after exposure to cyanide. The original purpose of this experiment was to ascertain whether or not cleavage of a disulfide bond (or bonds) was occurring as a result of treating the enzyme with cyanide. The results (shown in Table 5) indicate that the number of thiol groups had not increased as would be expected if a disulfide bond (or bonds) were broken, but actually decreased by one. These and other results discussed earlier were taken to indicate that the scission of a disulfide bond does not occur under these conditions. An alternate suggestion, supported by circular dichroism spectra, is that cyanide induces a small conformational change in the region of the active site of KHG-aldolase which allows the cyanide anion to bind electrostatically to a group which is now accessible in that region of the molecule. This cyanide-induced change in conformation might be somewhat the same as that produced by glyoxylate or pyruvate and, if so, would account for the previously unexplained decrease in the number of

thiol groups when one titrates the enzyme with DTNB. Just as substrates may induce a "tight" conformation of KHG-aldolase which makes 1 to 2 cysteinyl residues inaccessible for reaction with PMB in the native enzyme and with DTNB even in 8 M urea (51), so also in this experiment cyanide might be inducing a comparable "tight" conformation.

Although KHG-aldolase is inactivated during the course of being titrated with PMB, there appears to be no real correlation between the number of sulfhydryl groups reacted and the percentage of the initial enzymic activity remaining (Table 13). In fact, 2 to 3 sulfhydryl groups may be titrated before the enzyme undergoes any significant loss of catalytic activity. Even after the titration of 8 such groups, the enzyme still maintains 15% of its initial enzymic activity. These results contrast with those obtained with TNM-oxidation or with photooxidation of the aldolase. All enzymic activity is lost concomitant with the oxidation of 4 thiol groups by TNM. The effects of photooxidation are not as clear cut as those obtained with TNM due to the simultaneous loss of histidyl and cysteinyl residues in the former process. In any event, a significant loss of enzymic activity occurs before even one to two sulfhydryl groups are oxidized. Enzymic inactivation accompanying PMB titration most closely resembles that which occurs during the titration of KHG-aldolase with DTNB (51). The results seem to be most consistent with a general loss of the native conformation of the enzyme due to the

introduction of a bulky substituent onto thiol groups, rather than a consequence of the modification of specific groups essential for catalytic activity.

The total 1/2-cystine content of KHG-aldolase has been determined for the first time. A total of fourteen 1/2-cystine residues are present in the enzyme. Because 10 of these residues can be titrated as free thiol groups using PMB in the presence or absence of 1% SDS, KHG-aldolase is assumed to have two disulfide bonds in the molecule.

Data sufficient to substantiate the complete mechanism of action of KHG-aldolase has not yet been accumulated. Several points concerning the activity of the enzyme, however, can be made on the basis of data currently available. The enzyme catalyzes an aldol cleavage or condensation by means of an overall mechanism involving the intermediate formation of Schiff bases, and carbon-carbon bond synthesis or cleavage with concurrent proton exchange. These events appear to occur by distinct steps rather than in a concerted fashion. In addition to the lysyl residue(s) which actually participates in Schiff-base formation, this part of the mechanism appears to also require certain unmodified sulfhydryl groups in the enzyme. These sulfhydryl groups may be necessary for maintaining the proper steric relationship of groups around the active site either for an initial "recognition and binding" of the substrate or for actual Schiff-base formation.

Preliminary evidence is now available which indicates that histidyl residues may also play some function in the mechanism of KHG-aldolase. Suggested roles for this residue include: (1) providing positively charged sites in the enzyme for attracting the carboxylate anions of the substrate, (2) functioning as the amphoteric group responsible for proton abstraction and donation, and/or (3) serving in a capacity, like cysteinyl residues, of maintaining the integrity of the active site.

It is hoped that further studies on this aldolase will provide direct correlations between its structure and the mechanism whereby it accomplishes catalysis.

SUMMARY

Bovine liver KHG-aldolase is irreversibly inactivated by cyanide in the presence of glyoxylate, formaldehyde, or several of their carbonyl analogs. This inhibition has been shown to be due to the addition of cyanide to the double bond of a Schiff-base intermediate yielding a stable aminonitrile. The radioactive product obtained from acid hydrolysates of KHG-aldolase previously exposed to cyanide in the presence of ^{14}C -formaldehyde, has been isolated and characterized. When this radioactive product was examined by paper chromatography, it migrated identically with the expected product, N^6 -carboxymethyllysine, in five different solvent systems. These results represent the first instance in which an aminonitrile has been identified as being the product formed during substrate-dependent inhibition by cyanide of a Schiff-base mechanism enzyme. Kinetic studies show that this irreversible inactivation by cyanide in the presence of substrate is a biphasic process. These kinetic data are discussed in terms of a reaction scheme composed of three competing equilibria: (1) cyanide addition to the Schiff-base intermediate, (2) cyanohydrin formation between carbonyl substrate and cyanide, and (3) an interaction of cyanide with the enzyme which is not dependent on substrate. The nature of this unusual interaction between KHG-aldolase and cyanide has been the subject of several experiments. A strictly ionic interaction seems unlikely due to the slow rate of the reaction. Covalent attachments which were

considered, but rejected, were cyanohydrin formation with a carbonyl moiety in the aldolase, complexation of an essential metal ion, and cleavage of a disulfide bond in the enzyme molecule. Binding studies, using carbon-14 labeled cyanide, indicate that about 0.4 mole of cyanide is "bound" per mole of KHG-aldolase. At least 75% of this radioactive cyanide appears to be held by ionic forces. An explanation which could account for the slow rate of this apparent ionic interaction is proposed. It is suggested that cyanide may induce a small conformational change, at, or near, the active site. This change then allows for the ionic binding of the cyanide anion in a previously inaccessible region of the enzyme. The rate of this conformational change would then limit the rate of the ionic binding.

Photooxidation of KHG-aldolase in the presence of methylene blue results in a rapid and pseudo-first order inactivation of enzymic activity. The aldolase activity of the enzyme toward the D- and L-isomers of KHG as well as its β -decarboxylase activity with OAA as substrate are both lost at the same rate and to the same extent. Substrates for the enzyme provide little or no protection against this process of photoinactivation.

KHG at a concentration of 30 mM protects aldolase activity to the extent of 81%. Amino acid analyses of enzyme which is 98% photoinactivated show a loss of 22 out of 24 histidyl residues and 8 out of 10 cysteinyl residues in the molecule. Complete reaction of

all the sulfhydryl groups in KHG-aldolase with p-hydroxymercuribenzoate (PMB) does not prevent the cysteinyl residues from being destroyed during the process of photooxidation. The pseudo-first order rate constant for the loss of enzymic activity is identical to that for the destruction of histidyl residues. The rate constant for the destruction of cysteinyl residues is about one-half as large. The pH-rate profile for photoinactivation of KHG-aldolase is sigmoidal with an inflection point at about 7.3; increasing pH values result in an increased rate of inactivation. This rate profile as a function of pH is consistent with the photooxidation of either essential histidyl or cysteinyl residues in the protein molecule.

The K_m of the aldolase is unchanged by the process of photooxidation; the entire effect is reflected in a lowered value for V_{max} . Photooxidized KHG-aldolase is incapable of forming Schiff-base intermediates with its substrates, pyruvate and glyoxylate, even though the active-site lysyl residue is not destroyed. This result could be due to the loss of a group(s) essential for the initial recognition and binding of substrate prior to Schiff-base formation. Loss of the ability to form Schiff-base intermediates could also be attributed to a disruption of protein conformation which destroys the hydrophobic nature of the active site. Such hydrophobicity is thought to be responsible for the ability of the active-site lysyl residue to remain unprotonated at pH values of 7 to 8; it is the unprotonated form of this aminoacyl residue that participates in

Schiff-base formation during enzymic catalysis. Although it is very possible that the proton-exchange step in the mechanism of action of the aldolase is also destroyed during photooxidation, due to the loss of an essential histidyl residue, this would not be expected to affect the ability of the enzyme to form Schiff-base intermediates since KHG-aldolase is believed to catalyze aldol-type reactions by a stepwise, rather than a concerted, mechanism.

On the basis of the data presented in this thesis, it is not possible to attribute the loss of enzymic activity caused by photo-oxidation to the destruction of a specific residue (either histidyl or cysteinyl) in the protein. It is very likely that both aminoacyl residues are essential in order for the aldolase to function properly in catalysis.

Titration of KHG-aldolase using *p*-hydroxymercuribenzoate results in the reaction of 10 sulfhydryl groups. No direct correlation appears to exist between the number of such groups titrated and the observed concomitant loss of enzymic activity. Inactivation by *p*-hydroxymercuribenzoate seems to be more a function of general changes in conformation of the aldolase rather than the number of essential sulfhydryl groups titrated. Substrates (10 mM glyoxylate or 10 mM pyruvate) protect 1 to 2 sulfhydryl groups from reaction with *p*-hydroxymercuribenzoate. It is suggested that the binding of substrates induces a small conformational change in the enzyme which make these 1 to 2 sulfhydryl groups inaccessible for reaction

with *p*-hydroxymercuribenzoate. The occurrence of a protein conformational change is supported by previous data concerning the titration of KHG-aldolase with DTNB (51), and by data presented in this thesis on the number of sulfhydryl groups in the enzyme which can react with DTNB in the presence and absence of cyanide.

Amino acid analyses after acid hydrolysis of KHG-aldolase in the presence of DMSO indicate a total of 14 one-half cystine residues in the enzyme. By difference, native KHG-aldolase apparently contains 2 disulfide bonds.

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