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Inactivation of Bovine Liver 2-Keto-4-hydroxyglutarate Aldolase by Cyanide in the Presence of Aldehydes†

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ABSTRACT: Kinetic data show that the irreversible inactivation of liver 2-keto-4-hydroxyglutarate aldolase observed when the enzyme is incubated with an aldehydic substrate (or substrate analogue) in the presence of cyanide is a biphasic process and can, under certain conditions, involve a direct interaction between the enzyme and cyanide. The kinetic data are consistent with a scheme consisting of three competing reactions: (1) irreversible addition of cyanide to the enzyme-substrate Schiff base intermediate, (2) reversible cyanohydrin formation between cyanide and the aldehydic substrate (or substrate analogue), and (3) an interaction of cyanide with the enzyme which is not substrate dependent. Approximately 0.4 mol of cyanide is associated with 1 mol (120 000 g) of enzyme when 2-keto-4-hydroxyglutarate aldolase is incubated with [^{14}C]-cyanide followed by exhaustive dialysis; an ionic attachment, possibly at a carboxylate binding site, is suggested. Whereas native enzyme, not treated with cyanide, has ten Nbs_2-titratable sulfhydryl groups, approximately one less such group reacts with Nbs_2 when the aldolase is incubated with cyanide (in the absence of aldehydic substrates). It is suggested that the binding of cyanide results in a conformational change of the enzyme; conformational changes in the presence of cyanide are confirmed by circular dichroism spectra.

L-Hydroxyproline is a major constituent of collagen. The terminal step in the catabolism of this abundant amino acid in mammals, whereby the carbon chain is degraded to smaller fragments, is catalyzed by 2-keto-4-hydroxyglutarate aldolase (2-oxo-4-hydroxyglutarate glyoxylate lyase $\rightleftharpoons$ pyruvate + glyoxylate). This enzyme, which has been highly purified from extracts of rat liver (Rosso and Adams, 1967), bovine liver (Kobes and Dekker, 1969), and *Escherichia coli* (Nishihara and Dekker, 1972), catalyzes the dealdolization of kHOGlt,[1] yielding equimolar amounts of pyruvate and glyoxylate. Although the reaction is readily reversible, currently available information appears to indicate that the primary role of this aldolase in mammals is to catalyze kHOGlt degradation rather than its synthesis.

kHOGlt-aldolase from bovine liver is a Schiff base mechanism (class I) aldolase with a molecular weight of 120 000 (Kobes and Dekker, 1969, 1971a). As an aldolase, it is unusual in that it (a) binds not only kHOGlt and pyruvate but also glyoxylate via an azomethine linkage to the ϵ-amino group of an active-site lysyl residue (Kobes and Dekker, 1966, 1971a); (b) nonstereospecifically catalyzes the cleavage or formation of the D and L isomers of kHOGlt (Kobes and Dekker, 1971b); and (c) is 50% as effective as a β-decarboxylase toward oxalacetate as it is as an aldolase with kHOGlt (Kobes and Dekker, 1971b).

Cyanide is, for a variety of reasons, a versatile inhibitor of enzyme-catalyzed reactions; it readily combines with the metal ion which is essential for the activity of certain enzymes (i.e., cytochrome oxidase), it forms a cyanohydrin with carbonyl compounds that are required for enzyme activity (i.e., pyridoxal phosphate requiring enzymes), it causes slow but virtually irreversible inactivation of some enzymes by scission of essential disulfide linkages, and it also destroys xanthine oxidase activity by elimination of sulfur as thiocyanate from the protein (Massey and Edmondson, 1970). We previously reported that cyanide causes a substrate-dependent irreversible inactivation of kHOGlt-aldolase; no loss of activity is observed when the enzyme is incubated with kHOGlt or pyruvate in the presence of cyanide, but with glyoxylate (or other aldehydes, notably formaldehyde, glyoxal, or glycolaldehyde) a very rapid and irreversible loss of aldolase activity occurs (Kobes and Dekker, 1967; Hansen, et al., 1974). Working with either [^{14}C]cyanide or a ^{14}C-labeled aldehyde, we recently showed

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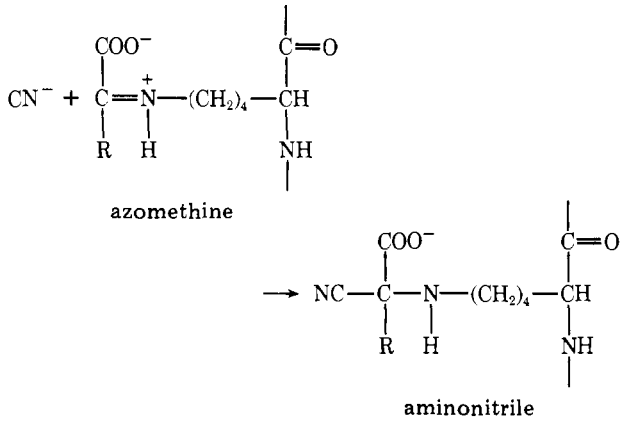
[1] Abbreviations used are: kHOGlt, 2-keto-4-hydroxyglutarate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); CD, circular dichroism; LDH, lactate dehydrogenase; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide.

unequivocally that inactivation is due, in this instance, to cyanide addition to the Schiff-base aldimine with stable formation of an aminonitrile (Hansen, et al., 1974).



The kinetics of this cyanide inactivation process are examined in this paper. The process is biphasic; cyanide apparently can interact directly with the aldolase and its presence protects one free sulfhydryl group in the enzyme molecule from reaction with Nbs_2.

Experimental Procedure

Materials. kHOGlt-aldolase was purified from extracts of bovine liver according to the procedure of Kobes and Dekker (1969). When required, some preparations of the enzyme were purified further on a column of DEAE-cellulose through which a diminishing concentration of ammonium sulfate was passed (Dekker, et al., 1975).

DL-kHOGlt was synthesized from *threo*-γ-hydroxy-DL-glutamate by nonenzymatic transamination, according to the procedure of Maitra and Dekker (1963). Acid hydrolysis of paraformaldehyde and subsequent distillation provided solutions of formaldehyde. Stock solutions of formaldehyde were standardized gravimetrically by precipitation with dimedon (Weinberger, 1931).

The following materials were obtained from the sources indicated: sodium glyoxylate and sodium dodecyl sulfate ("Ultrapure") from Sigma Chemical Co., glyoxal from Mann Research Labs., sodium [^{14}C]cyanide (5.28 mCi/mmol) from Nuclear-Chicago Corp., reagent grade sodium cyanide from Mallinckrodt Chemical Works, crystalline rabbit muscle lactate dehydrogenase (Grade A) in ammonium sulfate suspension from Calbiochem, its coenzyme, NADH, from P-L Biochemicals, Inc., and Nbs_2 from Aldrich Chemical Co. All other compounds were of the highest purity commercially available.

Methods. The concentrations of proteins in solution were routinely determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard; an auxillary method used in certain cases was that of Murphy and Kies (1960). Close agreement between these two methods was observed when applied to the same solutions.

Levels of radioactivity in solution were determined by liquid scintillation counting techniques using a Packard Tri-Carb Scintillation Spectrometer, Model 3320, with an efficiency of 73% for carbon-14.

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 automatically 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.

Two assays were used for the measurement of kHOGlt-aldolase activity. The first, a single-point assay, involved incubation of kHOGlt with the aldolase at 37 °C and colorimetrically determining the amount of glyoxylate formed after 20 min. This assay has been described in detail by Dekker and Maitra (1962).

The second is a rate assay in which pyruvate formed by cleavage of kHOGlt is utilized as substrate for LDH. The absorbance change at 340 nm accompanying the oxidation of NADH is monitored. The assay mixture contained the following components in a total volume of 1.0 ml: Tris-HCl buffer (100 μmol, pH 8.4), NADH (0.33 μmol), LDH (25–30 IU), kHOGlt (5 μmol), and an appropriate amount of enzyme. Addition of kHOGlt-aldolase initiated the reaction; the temperature was maintained at 26 °C.

The procedure used to titrate sulfhydryl groups with Nbs_2 was essentially that of Ellman (1959), except that higher concentrations of Nbs_2 and a slightly higher pH of reaction were used. A typical titration mixture contained kHOGlt-aldolase (0.20–0.30 mg), Tris-HCl buffer (50 μmol, pH 8.0), and Nbs_2 (0.5 μmol in 0.1 M potassium phosphate buffer, pH 7.0) in a final volume of 1.0 ml. In some instances, 1% sodium dodecyl sulfate was used as a denaturant. Nbs_2 was added last and the change in absorbancy at 412 nm 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 except aldolase was used to correct for small extraneous increases in absorption at that wavelength.

Results

Kinetics of the Irreversible Inactivation of kHOGlt-Aldolase Activity by Cyanide. Kinetic data were obtained by coupling the cleavage of kHOGlt with lactate dehydrogenase and measuring the disappearance of NADH at 340 nm (see Methods). The aldehydic substrate or substrate analogue was first incubated with the aldolase at 37 °C for 15 min to assure maximum azomethine formation. Thereafter, sodium cyanide was added to the mixture ($T = 0$) and aliquots were withdrawn at specific times. These aliquots were immediately diluted (500×) into the LDH assay system and the level of aldolase activity was determined. The results of such experiments are displayed in Figure 1. Inactivation of kHOGlt-aldolase activity by cyanide appears to be a biphasic process with formaldehyde and with glyoxal; in the presence of glyoxylate, the process is very rapid and essentially complete within 30 s. Other investigators (Cash and Wilson, 1966; Brand and Horecker, 1968) who observed a similar, although *reversible*, inhibition of fructose 1,6-bisphosphate aldolase and transaldolase by cyanide suggested that two competing equilibria might exist between cyanide, carbonyl substrate, and enzyme. The first of these equilibria is formation of the enzyme-substrate azomethine (a rapid process) followed by the addition of cyanide to the complex yielding an inactive aminonitrile. The second stage is cyanohydrin formation between cyanide and the carbonyl substrate. As applied to the *irreversible* inactivation of kHOGlt-aldolase by cyanide, the latter reaction would have the following consequences; it would (1) decrease the steady-state concentration of free substrate, (2) decrease the level of

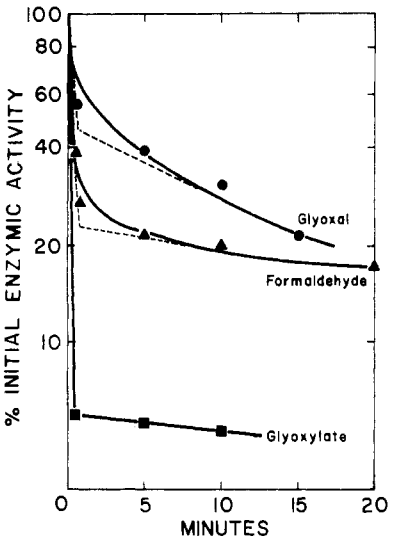


FIGURE 1: Kinetics of kHOGlt-aldolase inactivation by cyanide in the presence of aldehydes. kHOGlt-aldolase (300 μg), 2.25 μmol of aldehyde, and 50 μmol of potassium phosphate buffer (pH 7.4) were initially incubated for 15 min at 37 °C. Sodium cyanide (4.4 μmol) was then added (0 min on graph). The final reaction volume was 0.30 ml. At the times indicated, 2- or 5-μl aliquots were withdrawn, immediately diluted, and the level of aldolase activity determined by the coupled LDH assay. Observed activity remaining at indicated time (—); extrapolation of faster and slower rate (- - - -).

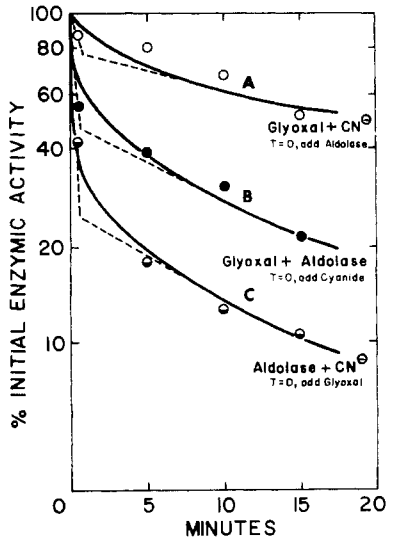


FIGURE 2: Effect on the inactivation kinetics of preincubating two different reaction components. The experiments were performed in the same manner as those described in Figure 1, with the exceptions noted. (●), Glyoxal and kHOGlt-aldolase were subjected to a prior incubation, as described and illustrated in Figure 1. This curve is included for reference. (○), The mixture initially incubated contained 4.4 μmol of sodium cyanide instead of enzyme. At 0 min, 300 μg of kHOGlt-aldolase was added and samples were withdrawn for assay at the times indicated. (◒), kHOGlt-aldolase and 4.4 μmol of sodium cyanide were initially incubated together. At 0 min, 2.5 μmol of glyoxal was added. Observed activity remaining at indicated time (—); extrapolation of faster and slower rate (- - - -).

available cyanide, and (3) slowly release both cyanide and substrate back into solution as the cyanohydrin dissociates. The rapid rate of inactivation observed initially, therefore, would be attributed to addition of cyanide to the preformed azomethine. The slower phase of the process is rate-limited by the breakdown of cyanohydrin. According to such a scheme, dissociation of the cyanohydrin is necessarily a slower reaction than subsequent formation of azomethine and aminonitrile.

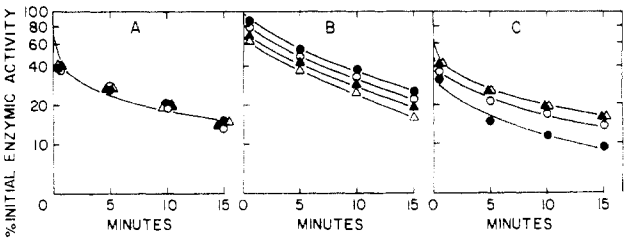
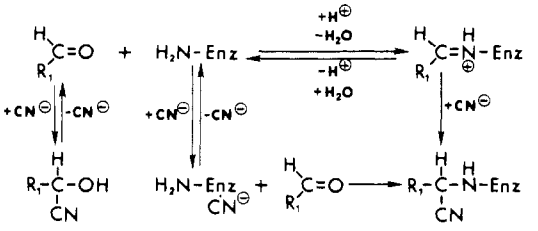


FIGURE 3: Effect on the inactivation kinetics of varying the time two different reaction components are preincubated. The experiments were performed in the same manner as those described in Figure 1, with the exceptions noted. The two components listed were subjected to an initial incubation: frame A, glyoxal and kHOGlt-aldolase; frame B, glyoxal and cyanide; frame C, kHOGlt-aldolase and cyanide. The time of preincubation in each case was 30 s (△), 1 min (▲), 5 min (○), and 15 min (●).

Effect of Subjecting Two Different Reaction Components to an Initial Incubation. If the foregoing concept of two competing equilibria applies, one would expect that the kinetic profile of inactivation could be changed by varying the two reaction components which are subject to a prior incubation. If, for example, cyanide and carbonyl compound are first incubated together in the absence of kHOGlt-aldolase, a much larger fraction of these two components would be present as the cyanohydrin (i.e., less available for azomethine and aminonitrile formation). If aldolase is then added, the extent of the rapid-phase inactivation should be greatly diminished. Glyoxal was chosen as the substrate for such studies, since the extent of the rapid reaction is smallest with this compound and both phases of the inactivation process can be followed conveniently. The results are shown in Figure 2.

When compared with curve B, the nature and extent of inactivation indicated by curve A are clearly in agreement with the model. An unexpected result is shown by curve C wherein cyanide and kHOGlt-aldolase are subjected to an initial incubation followed by the addition of glyoxal after the usual 15 min. It appears that in this case a third reaction must be considered. Cyanide apparently interacts with the aldolase in a manner which enhances the ability of this anion to react with the azomethine. The overall set of competing reactions involved in this case, therefore, is outlined in Scheme I.

SCHEME I: Competing Reactions Involved in the Inactivation of kHOGlt-Aldolase by Cyanide in the Presence of Aldehydes



Effect of Varying the Time of an Initial Incubation Period. In the experiments just described, two reaction components were initially incubated together for 15 min; thereafter, the third component was added to initiate the inactivation of aldolase activity. If the scheme of competing equilibria (Scheme I) is valid, the *length* of time two reaction components are subjected to an initial incubation should affect the extent of the rapid inactivation process. Experiments for this purpose were carried out as described in the legend of Figure 1 except the times of incubating each reaction couple were varied from 30 s to 15 min; the results are shown in Figure 3. As expected,

TABLE I: Interaction of [^{14}C]Cyanide with kHOGlt-Aldolase in the Absence of Any Aldehyde.[a]

Additions to kHOGlt-Aldolase	Further Treatment of Enzyme	Radioactivity Bound (cpm/μmol of protein)	[^{14}C]Cyanide Bound/ 120 000 μg of Protein (μmol)
[^{14}C]Cyanide (10 mM)	None	3.41×10^6	0.40
[^{14}C]Cyanide (10 mM)	Unlabeled NaCN (10 mM)	0.79×10^6	0.09
[^{14}C]Cyanide (10 mM)	0.5 M KCl	0.82×10^6	0.09

[a] The conditions of the experiment are described in the text.

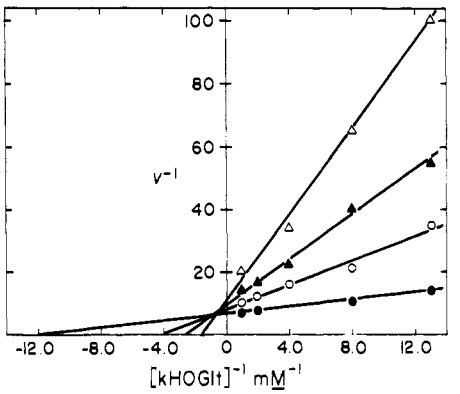


FIGURE 4: kHOGlt-aldolase activity in the presence of cyanide as a function of kHOGlt concentration. Enzymatic activity was determined by the colorimetric assay for glyoxylate; the usual time of incubation, however, was shortened from 20 to 10 min. In each assay, 0.7 μg of kHOGlt-aldolase was used. Upon dilution, the aldolase could be fully reactivated in each case. Either no cyanide was present (●), or 0.125 mM (○), 0.20 mM (▲), and 0.40 mM (△) cyanide was added.

the data in frame A establish that there is no apparent time effect on the establishment of a rapid equilibrium between kHOGlt-aldolase, glyoxal, and the aldolase-glyoxal Schiff base. Frame B shows the results when glyoxal and cyanide are first incubated together for varying intervals of time. After 15 min of incubation, a small degree of the rapid inactivation process is seen (about a 20% activity loss). As the time of incubating these two components is progressively shortened, however, somewhat more of the rapid inactivation reaction is seen each time until after only 30 s nearly 50% of the initial enzymatic activity is lost in the rapid phase. Qualitatively, these results are consistent with the proposed scheme of competing reactions—i.e., as the initial incubation time is shortened, less and less cyanohydrin is formed. It would also appear that cyanohydrin formation is a slower process than azomethine formation.

Frame C in Figure 3 shows the results when kHOGlt-aldolase and cyanide are first incubated together for varying time intervals. In this instance, *more* extensive rapid inactivation occurs after *longer* times of incubation. After 30 s, 70% of the initial enzymatic activity is rapidly lost as opposed to about 60% when cyanide and enzyme are not initially incubated together (frame A). After 15 min of incubation, however, the rapid inactivation reaction accounts for an 85% loss of the initial enzymatic activity. These data are also in agreement with the proposed scheme. Longer times of incubation allow for more extensive interaction between kHOGlt-aldolase and cyanide, thereby facilitating inactivation as soon as the Schiff base intermediate is formed. Here, again, the reaction of cyanide with the enzyme is apparently slower than azomethine formation.

Stoichiometry of Cyanide Association with kHOGlt-Aldolase in the Absence of an Aldehydic Substrate or Substrate Analogue. An attempt was made to ascertain whether or not any cyanide might actually become associated in some manner with the aldolase and, if so, how much.

kHOGlt-aldolase (0.797 mg), Tris-HCl buffer (200 μmol, pH 7.4), and sodium [^{14}C]cyanide (10 μmol, 5.28 μCi/μmol) were incubated for 1 h at 25 °C in a total volume of 1.0 ml. The solution was subsequently dialyzed against 1 l. of 0.01 M Tris-HCl buffer, pH 7.4, for 24 h. Two changes of dialyzing buffer (1 l. each) were made during this time. The dialyzed solution was assayed for kHOGlt-aldolase activity, protein content, and level of radioactivity. The moles of [^{14}C]cyanide bound/mol (120 000 g) of aldolase were computed (Table I). A measurable level of cyanide does, indeed, appear to be associated with the enzyme; the stoichiometry of this "binding" is about 0.40–1.0, cyanide to aldolase. Aldolase containing such "bound" cyanide is enzymatically fully active—i.e., no reduction in specific activity. In separate experiments, kHOGlt-aldolase solution from the above experiment which contained "bound" [^{14}C]cyanide was further dialyzed against either 1 l. 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 I, both of these treatments remove a significant fraction (about 75%) 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. The facile exchange of labeled, "bound" cyanide for either unlabeled cyanide or KCl argues for a type of ionic, as opposed to covalent, interaction between cyanide and the enzyme.

Cyanide as a Reversible Mixed-Type Inhibitor of kHOGlt-Aldolase Cleavage Activity. kHOGlt and pyruvate are organic acids having a carboxyl group (or groups); both compounds form an azomethine with the active-site lysyl residue of kHOGlt-aldolase and are required participants in the overall mechanism of the aldolase-catalyzed cleavage of kHOGlt (Kobes and Dekker, 1971a). Cyanide as an anion has molecular dimensions not unlike the carboxylate group of organic acids. It seemed possible, therefore, that cyanide might be specifically bound by ionic interaction at a carboxylate-binding site on the enzyme. If this occurred, competitive inhibition of aldolase activity by cyanide would be expected.

Inhibition is, indeed, observed (Figure 4); it is of a mixed competitive-noncompetitive nature. It is freely reversible by dilution and does not lead to irreversible inactivation. The K_i for cyanide determined in 0.1 M Tris-HCl buffer, pH 8.4, by the method of Dixon (1953) is 5.7×10^{-4}M.

Such inhibition studies were possible even though cyanide also irreversibly inactivates the aldolase; this occurs, however, only in the presence of *aldehydic* substrates (or substrate analogues). The unusual formation of an azomethine between the enzyme and glyoxylate (or other analogues) in this instance is considered to be "abortive" and is not required mechanistically (Kobes and Dekker, 1971a). Irreversible inactivation of enzymatic activity by cyanide is not observed in the presence of either kHOGlt or pyruvate (Kobes and Dekker, 1967; Hansen, et al., 1974). Therefore, aldolase actively involved in

catalyzing the cleavage of kHOGlt would for a time at least escape irreversible cyanide inactivation, especially when initial enzyme rates are measured for at such times the glyoxylate concentration is not large enough to significantly compete with kHOGlt for the active-site lysyl residue.

Sulfhydryl Content of kHOGlt-Aldolase after Exposure to Cyanide in the Absence of an Aldehyde. Lane et al. (1976) found that bovine liver kHOGlt-aldolase has 8–10 free sulfhydryl groups which can be titrated with either Nbs_2 or *p*-mercuribenzoate in the presence of 1% sodium dodecyl sulfate. The substrates, pyruvate or glyoxylate, protected one of the sulfhydryl residues from reaction with Nbs_2; either substrate also prevented titration of 1–2 thiol groups by *p*-mercuribenzoate. In view of such experiments as well as the results reported here (i.e., a substrate-independent interaction of cyanide with the aldolase, plus mixed-type inhibition of kHOGlt-aldolase activity by cyanide), the effect that cyanide might have on the titration of sulfhydryl groups was examined.

kHOGlt-aldolase (0.525 mg), potassium phosphate buffer (100 μmol, pH 7.4), and sodium cyanide (4.5 μmol) were incubated for 15 min at 25 °C in a total volume of 1.0 ml. The solution was then dialyzed exhaustively against three 1-l. volumes of 0.01 M Tris-HCl buffer, pH 8.0, containing no thiol. The dialyzed solution was assayed for protein content as well as for the total number of free sulfhydryl groups in the enzyme molecule by titration in 1% sodium dodecyl sulfate with Nbs_2. Native enzyme, not treated with cyanide, showed 10.1 Nbs_2-titratable sulfhydryl groups, whereas aldolase incubated with cyanide always gave values within the range of 8.8–9.3. It would appear, therefore, that approximately one less sulfhydryl group is free for reaction in kHOGlt-aldolase containing "bound" cyanide than in the native enzyme.

Circular Dichroism Spectra of kHOGlt-Aldolase in the Presence and Absence of Cyanide. The possibility existed that cyanide interaction with kHOGlt-aldolase resulted in a conformational change of either a gross or local nature. In order to investigate this possibility, CD spectra were recorded on the enzyme in the presence and absence of cyanide. The CD cell contained kHOGlt-aldolase (1.50 mg for the 250–350-nm region; 0.033 mg for the 200–250-nm region), Tris-HCl buffer (100 μmol, pH 7.4), and sodium cyanide (15 μmol, 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 kHOGlt-aldolase from 200 to 250 nm exhibited a double minimum at 210 and 220 nm indicating the presence of α-helical structure in the enzyme. The spectrum recorded in 15 mM cyanide over the same region (200–250 nm) was identical to that of native aldolase indicating that no gross conformational changes are induced in the presence of this concentration of cyanide.

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

Discussion

A few reports have occurred recently in which specific interaction of cyanide with proteins has been noted. For example, Gaylord, et al. (1970) isolated a cyanide-binding protein from rat liver microsomes and Lorimer, et al. (1974) reported the presence of bound cyanide in the naturally inactivated form of nitrate reductase of *C. vulgaris*. Certainly, there are probably few substances that exhibit as many and as diverse effects in biological systems as does cyanide; inhibitory effects of cyanide on isolated enzyme systems are well documented. Some of these effects, to mention a few, are the consequence of metal ion complexation (notably Cu^{2+} and Fe^{3+}), scission of disulfide bonds, reaction with carbonyl groups, and addition to certain double bonds. Because of these multiple possible effects, it perhaps is not surprising to find that a given enzyme is subject to more than one type of interaction with cyanide.

That seems to be the case with kHOGlt-aldolase. Because of the observed *irreversible* inactivation of kHOGlt-aldolase by cyanide only in the presence of aldehydic substrates or substrate analogues (Hansen, et al., 1974), we recently were able to obtain first experimental proof that cyanide inactivation of enzymes that function via a Schiff base mechanism, not involving pyridoxal 5′-phosphate, is due to aminonitrile formation (i.e., cyanide adds to the Schiff base formed between the carbonyl substrate and the ε-amino group of a lysyl residue in the enzyme). For kHOGlt-aldolase, the kinetics of this inactivation are biphasic. In previous studies with cyanide plus transaldolase (Brand and Horecker, 1968), such biphasic inactivation kinetics were explained as involving two competing equilibria. This explanation seems applicable here, too, but in the instance of kHOGlt-aldolase yet a third competing equilibrium must be added, namely, a direct interaction of cyanide with the free aldolase. The overall set of reactions involved in this case, then, is as outlined in Scheme I.

In order to account for the observed kinetics, it seems most likely that the direct interaction of cyanide with kHOGlt-aldolase is a reversible process 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, something other than a strictly ionic attraction would at first seem to be indicated. Some of the known reactions of cyanide can be discounted on the basis of facts already known about kHOGlt-aldolase. For instance, complexation by cyanide of a metal ion does not seem applicable, since this aldolase does not appear to contain any metal nor is a metal ion apparently required for enzymatic activity (Kobes and Dekker, 1969). In addition, kHOGlt-aldolase has no essential carbonyl residue in the molecule, since no loss of enzymatic activity is observed after it is treated with borohydride in the absence of a substrate. 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 kHOGlt-aldolase contains such a residue. Cleavage of a disulfide bond in the enzyme molecule by reaction with cyanide does not appear likely either. Experiments in which the number of sulfhydryl groups present in kHOGlt-aldolase was determined before and after exposure to cyanide indicate no increase as would be expected in the event of disulfide bond cleavage. In addition, it should be recalled that incubation of the aldolase with cyanide in the absence of a carbonyl substrate followed by exhaustive dialysis results in no loss of enzymatic activity. Although not absolutely impossible, it is difficult to envisage the cleavage of one or two disulfide bonds in a protein occurring with complete retention of its enzymatic activity.

A total of 0.4 mol of radioactive cyanide/mol of aldolase is

held by direct interaction with the protein. Some 75% of this radioactivity is exchangeable with unlabeled cyanide or can be removed by dialyzing the protein solution exhaustively against a solution of 0.5 M KCl. Such behavior is most consistent with ionic, rather than covalent, attachment of cyanide to kHOGlt-aldolase. Possibly, cyanide interacts with a positively charged center on the enzyme. As a result, a small conformational change in the protein sequesters some cyanide to some extent from the aqueous media. Such a positive center might be one which normally binds an anionic carboxylate group of the substrate, kHOGlt; occupation of this site by cyanide would still allow binding as well as azomethine formation between kHOGlt-aldolase and 1- or 2-carbon aldehydes. In a ternary complex of this sort, the position of cyanide could be highly advantageous for ready addition to the -C=N- of the azomethine to yield an aminonitrile. Enhanced cyanide inactivation following preincubation of kHOGlt-aldolase with cyanide would then be considered a result of such ternary complex formation.

Interaction of cyanide with a positively charged center on the enzyme which normally is an anionic carboxylate binding site might have a very different effect on the binding of kHOGlt; competitive inhibition would seem most likely. A mixed competitive-noncompetitive type of inhibition on kHOGlt aldolytic activity is actually observed; this is independent of irreversible aminonitrile formation which does not occur with either kHOGlt or pyruvate as the carbonyl compound (Hansen, et al., 1974). An inhibition pattern of this nature is not unreasonable so long as allowance is made for additional inhibitory interactions besides cyanide blocking of an anionic carboxylate binding site. The data in Table I, in fact, suggest that two different types of interactions might possibly be involved between cyanide and the enzyme. Of the total 0.4 mol of radioactive cyanide held by interaction with the protein, only 0.31 mol or 75% is quite readily exchangeable. The remaining 0.09 mol routinely remains in association with the enzyme and is assumed to be held in a different manner.

When kHOGlt-aldolase is first treated alone with cyanide and then subsequently reacted with Nbs_2, one less sulfhydryl group (nine SH groups rather than ten total) is available for titration than with enzyme that has been handled identically in the absence of cyanide. This result is interesting in view of the recent paper of Lane et al. (1976) where it is reported that incubation of kHOGlt-aldolase with either pyruvate or glyoxylate also renders one to two sulfhydryl groups in the enzyme unreactive toward Nbs_2 or *p*-mercuribenzoate. It was not suggested that either glyoxylate or pyruvate chemically protected the unreactive sulfhydryl groups; rather, it was postulated that binding of one of the substrates at the active site of the aldolase induced a conformational change in which one to two sulfhydryl groups become unavailable to titrants even in the presence of common denaturants. It now appears that cyanide, also possibly interacting with a binding site, causes a similar conformational change in the enzyme which affects the reactivity of a sulfhydryl group toward Nbs_2. A slight conformational change of the enzyme in the presence as opposed to the absence of cyanide has been confirmed by circular dichroism spectra.

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