Hydroxamic Acid Production by α-Ketoglutarate Dehydrogenase. Part 2.[1] Evidence for an Electrophilic Reaction Intermediate at the Enzyme Active Site

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The α-ketoglutarate dehydrogenase-catalyzed conversion of 4-chloronitrosobenzene (1) into the hydroxamic acid (3) and the Bamberger-rearrangement product (6) was investigated by use of radiotracer methods and nucleophilic trapping agents. ^{14}C-Labelled 4-chloronitrosobenzene (1) failed to give any significant incorporation of radiolabel into the protein of the enzyme, or into calf thymus DNA. The production of a third and highly polar metabolite during this reaction was confirmed; however, the structure of this metabolite has not been elucidated. In the presence of high concentrations of halide salts, the product distribution for the enzymic reaction was markedly altered. In the order $I^- > Br^- > Cl^-$, halides inhibited the production of the rearrangement product (6) and of the unknown polar product. The inhibition of the formation of these products was accompanied by a considerable increase in the amount of hydroxamic acid (3), and by the production of a new metabolite, the structure of which was dependent upon the halide employed in the reaction. In the case of both Br^- and Cl^-, the new metabolite [(8a) and (8b), respectively] was indicative of the trapping of an enzyme-generated electrophile by halide anion. In the case of I^-, the initial trapping of the electrophilic species was followed by a redox process to give 4-chloroaniline (9).

The expected ability of α-ketoglutarate dehydrogenase (α-KGD) to convert a nitroso-aromatic compound (1) into the *N*-succinyl-derived hydroxamic acid (3) was recently reported.[1] An unusual side reaction that was discovered during that investigation is the ability of α-KGD to also catalyze a Bamberger-like rearrangement reaction with resultant production of the isomeric *o*-hydroxyamide (6). In contrast, other thiamine-dependent enzymes, including transketolase [2,3] and pyruvate decarboxylase,[3,4] have not been found to catalyze a Bamberger-like rearrangement in the course of hydroxamic acid production. This unique property of α-KGD prompted us to investigate the possible production of an electrophilic species by the action of this enzyme on nitroso-aromatics.

Trapping experiments with organic nucleophiles such as aniline were not successful in elucidating the existence of a reactive intermediate during the enzymic reaction. In a subsequent experiment, we found that a high concentration of inorganic bromide ion had very significant effects upon the course of this enzymic reaction. We now report on this effect, along with similar effects by I^- and Cl^-. The results of this study support our original hypothesis that a reactive species is produced at the active site of the enzyme.

It was shown previously that the combined yield of identifiable products resulting from the action of α-KGD on 4-chloronitrosobenzene (1) was not quantitative. A total of about 50% of the substrate was found to be converted into the hydroxamic acid (3) and the rearrangement product (6).[1] The fate of nearly half the substrate was unknown, although evidence suggested that either protein binding or the production of a highly water-soluble metabolite was the cause of the poor material balance.[1] In the present study, ^{14}C-labelled (1) was employed in an attempt to elucidate more fully this enzymic reaction. In addition to confirming our initial observation that (1) was converted into (3) and (6) in a combined yield of nearly 50%, these radiotracer experiments showed that the balance of uncharacterized product consisted of a highly polar metabolite of (1). The polar nature of the unidentified product(s) was concluded on the basis of high pressure liquid chromatography (h.p.l.c.) studies of the radiolabelled reaction product. The unidentified portion of the reaction product was found to elute rapidly through a reverse phase h.p.l.c. column without resolution from the enzyme cofactor, thiamine pyrophosphate, and enzyme substrate, α-ketoglutaric acid. This polar product was found not to be associated with protein through covalent bonding, since trichloroacetic acid-precipitated protein did not contain any significant radioactivity above that of the controls. This finding demonstrated that incorporation of (1) into the protein structure of α-KGD was not the cause of the poor material balance.

The enzymic reaction with ^{14}C-labelled (1) in the presence of calf thymus DNA failed to indicate any incorporation of ^{14}C into DNA above that of the controls, which consisted of conducting the incubation in the absence of either α-ketoglutaric acid or α-KGD. Both the complete incubation and the two controls indicated that *ca.* 0.3% of the ^{14}C-labelled (1) was incorporated into DNA by processes independent of the action of α-KGD on (1).

In spite of the failure to observe significant binding of the substrate (1) to protein or DNA during the enzymic reaction, trapping experiments were conducted with other nucleophilic reagents in an attempt to trap the suspected reactive intermediate. The results of these trapping studies were largely unexpected, yet have proved to be of value in our attempt to understand the unusual chemical events that occur at the active site of α-KGD during reaction with the nitroso-substrate (1). The halides I^-, Br^-, and Cl^- caused major changes in the course of the reaction of (1) with the α-KGD enzymic system. These halide ions caused a concentration-dependent increase in the production of the hydroxamic acid product (3) (Figure 1), and a decrease in the production of the rearrangement product (6). These effects were greatest with I^- and least with Cl^-. At a concentration of I^- of 500mM, the percentage conversion of (1) into (3) increased from 33 to 84%, while the amount of (6) produced decreased from 15 to just 2%. Bromide (500mM) increased the amount of (3) produced from 33 to 72%, and decreased that of (6) from 15 to 5%, while chloride (500mM) increased the production of (3) from 33 to 57%, and decreased that of (6) from 15 to 9%. The combined yields of (3) and (6) varied from 86 to 76 and 65% for 500mM concentrations of I^-, Br^-, and Cl^-, respectively. Control experi-

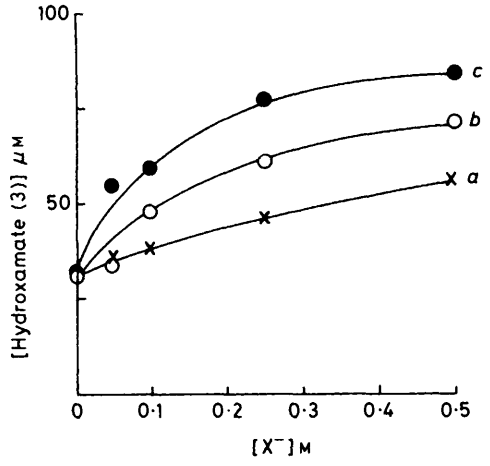


Figure 1. The effect of halides on the total enzymic conversion of the *nitroso*-benzene (1) into the hydroxamic acid product (3). The indicated concentration of KX, 4-chloronitrosobenzene (1) (0.098 μmol), α-KGD (0.1 unit), α-ketoglutaric acid (2 μmol), thiamine pyrophosphate (0.2 μmol), and $MgCl_2$ (1 μmol) in a total vol of 1.0 ml of 0.05M-KH_2PO_4 buffer (pH 7.5) were used. After 2 h [>5 half-lives of (1)] the reaction mixtures were analyzed by direct injection of 10-μl aliquots into the h.p.l.c. system, and the amount of (3) present was determined as described in the Experimental section. (*a*) Cl^-; (*b*) Br^-; (*c*) I^-

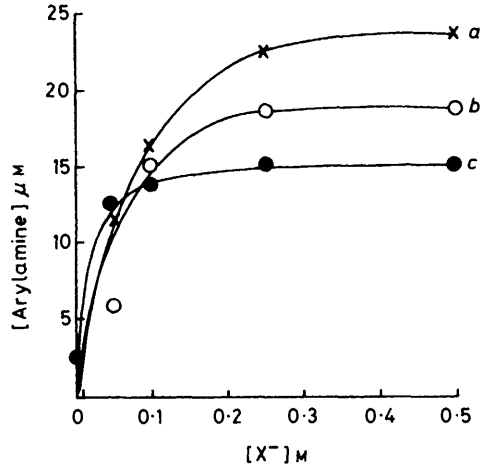


Figure 2. The effect of halides on the total enzymic conversion of compound (1) into the respective arylamine product. The reaction compositions and analytical methods were identical with those described in the legend to Figure 1. (*a*) Cl^-, arylamine (8b); (*b*) Br^-, arylamine (8a); (*c*) I^-, arylamine (9)

ments with the weakly nucleophilic sulphate anion under isoionic conditions demonstrated that nonspecific ionic effects were not responsible for the observed changes in product distribution.

Azide ion (250mM) increased the enzymic production of the hydroxamic acid (3) from 36 to 71%, while decreasing the production of (6) from 16 to 3%. On the other hand, cyanide ion was found to react rapidly with the substrate (1) in the absence of α-KGD to give unidentified products. Fluoride ion had no effect on the α-KGD reaction.

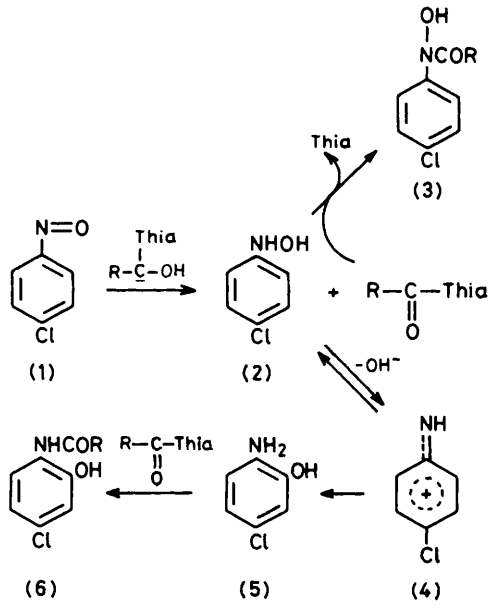
In addition to causing a marked increase in the conversion of (1) into (3), Br^- and Cl^- also caused the production of the corresponding ring-halogenated products (8a) and (8b). In the case of I^-, the ring-halogenated product was not observed; however, I^- did cause a significant increase in the amount of 4-chloroaniline (9) observed in the reaction mixture. Iodide (500mM) resulted in 15% of (1) being converted into (9), which was present only in trace amounts (1—2%) in the normal enzymic reaction. The ring-halogenated products (8a) and (8b) were identified in enzymic reactions containing Br^- or Cl^- by a comparison of their chromatographic properties with those of authentic standards. The succinic acid amides of (8) and (9) were synthesized, and shown not to be produced during the enzymic reaction in the presence of the appropriate halide salt. The concentration-dependent production of (8) and (9) is illustrated by Figure 2. The maximum amount produced of these third identifiable metabolites was 26% of (8b) in the case of Cl^- and 20% of (8a) in the case of Br^-. It is noteworthy that halide concentrations of 500mM increased the sum of identifiable metabolites to 100, 95, and 90% for I^-, Br^-, and Cl^-, respectively.

In contrast to the effect of halide ions upon product distribution, I^- had no effect on the overall rate of the enzymic reaction. Although the reaction rates for the formation of (3) and (6) changed in the presence of halide, the rate of disappearance of (1) was not affected.

Several related mechanisms had been proposed to explain the occurrence of the Bamberger-rearrangement product (6) during α-KGD-catalyzed conversion of (1) into the hydroxamic acid (3).[1] The effects of the halides I^-, Br^-, Cl^- on this enzymic reaction are inhibition of the formation of the rearrangement product (6), a marked increase in the production of the hydroxamate (3), and the appearance of a third identifiable metabolite. These halide effects can be explained on the basis that a halide ion reacts with an enzyme reaction intermediate that normally gives rise to the rearrangement product (6) and the as yet unidentified polar metabolite(s). The production of the ring-halogenated metabolites (8a) and (8b) is direct evidence that Br^- and Cl^- act as trapping agents for a reactive intermediate. The production of 4-chloroaniline (9) in the presence of I^- is also explained on the basis of an initial trapping reaction by I^-. The marked increase in the amount of hydroxamic acid (3) produced in the presence of these halides can also be rationalized on the basis of an initial nucleophilic trapping of an enzyme intermediate. These effects of the halides can be explained by each of the mechanistic possibilities that were originally proposed.[1] Thus, these studies do not allow us to differentiate between the possible mechanisms, but they do provide strong evidence for the production of a transient reactive intermediate at the active site of α-KGD during reaction with the nitroso-aromatic substrate (1).

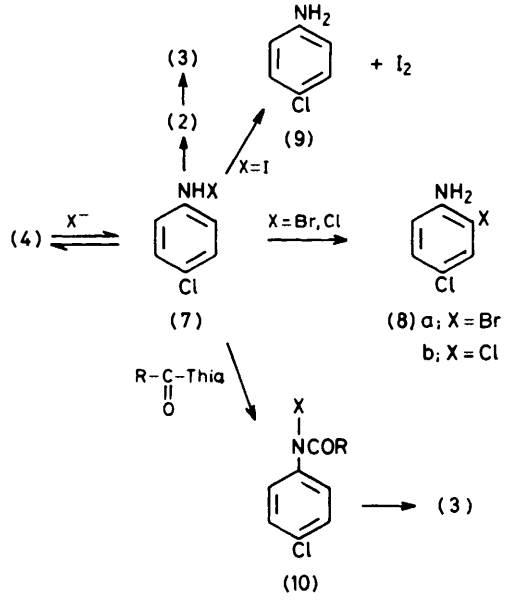
The initial event in the the reaction of the nitroso-substrate (1) with the α-KGD system is most probably a redox reaction with active-site bound ' active succinic semialdehyde ' (Scheme 1). The products of this reaction are the hydroxylamine (2) and ' active succinate ', the latter being a reactive acylating agent.[5] Production of the hydroxamic acid (3) is the result of condensation of these two products at the active site of α-KGD. Alternatively, a Bamberger rearrangement at the active site of α-KGD, followed by succinylation of the aminophenol (5), gives the observed product (6). This rearrangement reaction was proposed to proceed through the intermediate production of an electrophilic species such as (4), which explains not only the occurrence of the rearrangement product (6), but also the production of the as yet unknown polar metabolite(s).[1] The trapping of the intermediate (4) by a halide anion provides an explanation for the results of the present study.

Scheme 2 illustrates our proposal that the halides I^-, Br^-, and Cl^- trap the reactive intermediate (4) by covalent-bond formation with the nitrogen atom to give the *N*-halogenoamine (7). The ring-halogenated products (8a) and (8b) result from the known rearrangement reaction of *N*-halogenoamines.[6] Succinylation of such *ortho*-halogenated arylamines



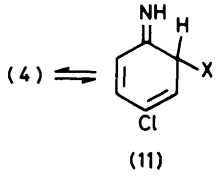
R = $CH_2CH_2CO_2H$ Thia = Thiamine

Scheme 1



R = $CH_2CH_2CO_2H$ Thia = Thiamine

Scheme 2



Scheme 3

was not observed in the enzymic reaction, and this is probably due to the steric hindrance present in compounds (8a) and (8b). This proposal does not preclude the possibility of direct nucleophilic substitution of halide ion onto the aromatic ring through the intermediate (4). In that case, the intermediate (11) would result from an initial trapping of (4) (Scheme 3). In the case of I^-, no ring halogenation was observed, but instead a rather significant production of 4-chloroaniline (9) occurred. Such a production of (9) is a reductive process, and is explained by iodide reduction of the *N*-iodoamine (7; X = I) or a similar reduction of the σ-complex (11; X = I). This explanation is consistent with a prior report that arylhydroxylamines [*e.g.* (2)] react with HI to give the arylamine [*e.g.* (9)], and that no ring-iodinated products result.[7] Attempts to detect I_2 as a co-product in the enzymic reaction were unsuccessful, since the minimum detectable limit for iodometric titrations [8] is larger than the amount of free I_2 expected during the enzymic reaction.

A compelling reason to propose the intermediate production of the *N*-halogenoamine (7) during the trapping reactions with halide ions was the interesting observation that all the halides increased the amount of the hydroxamic acid product (3). Scheme 2 illustrates this increased production of (3) as a result of the intermediate formation of the *N*-halogenoamine (7). Selective hydrolysis of (7) to give the hydroxylamine (2) (Scheme 2) would have the overall effect of increasing the active-site concentration of (2) at the expense of the proposed reactive intermediate [*e.g.* (4)]. Such an effect could explain the greatly increased production of the hydroxamic acid (3). Alternatively, *N*-succinylation of the halogenoamine (7) (Scheme 2) would result in the *N*-halogenoamide (10). Although (10) was not observed as a product of this enzymic reaction, its formation and selective hydrolysis to give (3) could also reasonably explain the facilitated production of (3). The chemistry of N–halogen bonds, particularly in aqueous solution, is poorly documented, and clear precedence for such conversion of N–halogen bonds into N–OH bonds is lacking. As illustrated by Figure 1, the order $I^- > Br^- > Cl^-$ was observed for the facilitated production of the hydroxamic acid (3). Such an order is expected for the trapping of a cation such as (4) by halides on the basis of their relative strengths as nucleophiles.[9] The suppression of the rearrangement reaction which gives (6) is thus a result of the trapping of the cation (4). The only remaining point of uncertainty is an explanation as to why more compound (3) is produced in the presence of a halide, when ring halogenation to give (8a), (8b), or (9) is the only expected result from the trapping of (4).

A totally different explanation can account for the unusual effect of halide ions upon this enzymic reaction. As originally proposed,[1] the production of the Bamberger-rearrangement product might be due to a Lewis-acid catalyst at the active site of α-KGD. Cl^-, Br^-, and I^- (in order of increasing extent) can co-ordinate this Lewis-acid site and mask its normal action on (2); inhibition of the formation of (6) is thus expected. In turn, more hydroxamic acid product (3) would be a likely consequence since diversion of (2) to the rearrangement reaction is inhibited.

The effect of halide ions on the α-KGD action on the nitroso-aromatic substrate (1) is further evidence for the production of a reactive chemical species at the enzyme active site. The observation that high concentrations of halide are required to fully observe this effect is consistent with the failure to observe incorporation of radiolabel into either enzyme protein or DNA. We conclude that the reactive species generated from 4-chloronitrosobenzene (1) is closely associated with the active site of α-KGD, and is thus unable to react readily with exogenous nucleophiles that are excluded for steric reasons.

Experimental

High pressure liquid chromatography (h.p.l.c.) was conducted by modification of a previously developed procedure.[10] U.v. spectra were obtained with a Beckman Model 35 spectrophotometer and i.r. spectra were obtained with a Nicolet 7199 FT-IR. N.m.r. spectra were obtained on a Varian EM-360 (60 Hz) or Nicolet NT-300 (300 Hz) spectrometer. Liquid scintillation counting was achieved by use of a Searle Analytic 92 liquid scintillation counter. M.p.s were obtained with a calibrated Thomas-Hoover m.p. apparatus and elemental analyses were performed by Galbraith Laboratories. The preparation of α-KGD was achieved by previously cited methods [1] from *E. coli*. 2-Bromo-4-chloroaniline (8a) was obtained from ICN Pharmaceuticals; 4-chloroaniline (9) and 2,4-dichloroaniline (8b) were obtained from Aldrich Chemical Co.

Synthesis of N-(4-*Chlorophenyl*)*succinamic Acid*.—To a solution of succinic anhydride (12 g, 0.12 mol) in dimethyl formamide (20 ml) was rapidly added a solution of 4-chloroaniline (9) (12.8 g, 0.10 mol) in Et_2O (100 ml). The reaction was stirred at room temperature for 1 h, then treated with H_2O (10 ml) and stirred for an additional 15 min. The reaction mixture was combined with Et_2O (250 ml), extracted twice with 50 ml of H_2O, dried (Na_2SO_4) and evaporated. The residue was decolourized (Norit neutral) and crystallized from aqueous acetone. Recrystallization from aqueous acetone gave the *succinamic acid* (14.6 g, 64%) as white plates, m.p. 171—172 °C (Found: C, 52.95; H, 4.55; N, 6.0. $C_{10}H_{10}ClNO_3$ requires C, 52.74; H, 4.43; N, 6.16%); $\nu_{max.}$ (KBr) 1 700 and 1 680 cm^{-1}; δ_H[$(CD_3)_2SO$] 10.2 (1 H, br s), 7.60 (4 H, sym. m), and 2.60 (4 H, br s).

Synthesis of N-(2,4-*Dichlorophenyl*)*succinamic Acid*.—By use of a procedure similar to the last, 2,4-dichloroaniline (8b) (16.2 g, 0.10 mol) was converted into the succinamic acid. Recrystallization (aqueous acetone) gave white needles (13.5 g, 52%), m.p. 150—151 °C (Found: C, 45.7; H, 3.5; N, 5.6. $C_{10}H_9Cl_2NO_3$ requires C, 45.81; H, 3.46; N, 5.35%); $\nu_{max.}$ (KBr) 1 695 and 1 660 cm^{-1}; δ_H[$(CD_3)_2SO$] 9.65 (1 H, br s), 7.90—7.30 (3 H, m), and 2.60 (4 H, br s).

Analytical Determinations on the Enzymic Reaction.—The usual methodology for the incubation of 4-chloronitrosobenzene (1) with the enzyme consisted of combining all the reactants except the enzyme in the 0.05M-KH_2PO_4 buffer (pH 7.5) and then adding the enzyme in 0.02M-KH_2PO_4 (pH 7.0) (reactant buffer–enzyme buffer, 4 : 1) to initiate the reaction. The enzyme substrate, α-ketoglutaric acid, was present at a concentration of 2.0mM, and the cofactors $MgCl_2$ and thiamine pyrophosphate were present at reaction concentrations of 1.0mM and 0.2mM, respectively. Anionic trapping agents (Cl^-, Br^-, I^-) were added as their potassium salts to the buffer. The nitroso-substrate (1) was added just before the enzyme solution as a concentrated solution in 95% EtOH to give a reaction concentration of 0.075—0.10mM. The amount of α-KGD activity was *ca.* 0.1 units per ml. In most cases, the final incubate volume was 1.0 ml. For h.p.l.c. analysis, 10 or 20 μl aliquots were directly injected onto a μBondapak C_{18} column and chromatographed with 50% methanol, buffered to pH 3.5 with 0.01M-KH_2PO_4, which contained 0.01% (w/v) of desferal mesylate. The solvent flow-rate was 1.5 ml min^{-1} and component detection was made at λ 254 nm. Quantitative calculations were made on the basis of component peak heights compared with peak heights generated by known amounts of authentic standards.

* The synthesis of [U-^{14}C]-4-chloronitrosobenzene will be published at a later date. Details are available from author.

Identification of Products (8a), (8b) *and* (9) *in Enzymic Reactions*.—Authentic samples of possible enzymic reaction products (8a), (8b), (9) and the succinamic acid derivatives of each were employed to determine their h.p.l.c. characteristics in several solvent systems. The identities of peaks present in the chromatograms of enzymic reactions containing I^-, Br^-, and Cl^- were then assigned by comparison with the chromatographic properties of the authentic standards. Assignments were made on the basis of identical retention times and identical peak-height ratios determined simultaneously at two different wavelengths by use of a Waters Model 440 dual wavelength absorbance detector. Solvents employed on a μBondapak C_{18} column were 60% MeOH and 50% acetonitrile; peak-height ratios were determined for λ254/λ313 in each solvent.

Study of the Binding of ^{14}C-*Labelled* (1) *to Enzyme Protein*.—Enzymic incubations were conducted in the usual manner on 1.0-ml reaction volumes containing a total of 260 μg of α-KGD. Controls consisted of the complete system, but lacking α-ketoglutaric acid. [U-^{14}C]-4-Chloronitrosobenzene * was present in the incubation solutions at a concentration of 0.078mM (0.05 μCi). After 2 h, protein was precipitated with trichloroacetic acid,[11] emulsified in 100 μl of Protosol (New England Nuclear) and counted as a solution in 15 ml of Aquasol 2 (New England Nuclear).

Study of the Binding of ^{14}C-*Labelled* (1) *to DNA*.—Enzymic incubations were conducted in the usual manner on 5.0-ml reaction volumes, but these also contained 2 mg ml^{-1} of calf thymus DNA (Sigma Chemical Co.). Controls had the same composition, but lacked either α-ketoglutaric acid or α-KGD. [U-^{14}C]-4-Chloronitrosobenzene was present in the incubation solutions at a concentration of 0.096mM (3.0 μCi). After 2 h, h.p.l.c. analysis was used to confirm production of compounds (3) and (6), and the DNA was then isolated by a modification of standard procedures.[12] The enzymic reaction and controls were extracted twice with an equal volume of phenol, and three times with Et_2O, and then precipitated twice with 3 vol of cold 95% EtOH. The resulting DNA pellet was dissolved in H_2O (5.0 ml) and analyzed for DNA by the diphenylamine method.[13] Aliquots of 1.0 ml each were hydrolyzed with HCl and counted in Aquasol 2 (12 ml).

Acknowledgements

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