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EFFECT OF RING SUBSTITUENTS ON THE TRANSKETOLASE-CATALYZED CONVERSION OF NITROSO AROMATICS TO HYDROXAMIC ACIDS

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Abstract—Transketolase catalyzed the conversion of eight different aromatic C-nitroso compounds into the corresponding N-glycolyl derived hydroxamic acids. Three of the nitroso compounds were also found to be converted in part to the arylhydroxylamines by a reductive process. A correlation was found for the rates of production of these metabolites with the electronegativities of substituent groups that were present on the aromatic ring. The rates of reaction of these substituted nitroso substrates with transketolase and D-fructose-6-phosphate were found to decrease in the order 4-NO_2 >> 4-CF_3 > 3-CF_3, unsubstituted > 4-Cl > 4-CH_3, 4-phenyl > 4-OC_2H_5. *N*,*N*-Dimethyl-*p*-nitrosoaniline was not metabolized by transketolase under the conditions employed for the other substrates. Those substrates possessing the strong electron-withdrawing groups 4-NO_2, 4-CF_3 and 3-CF_3 were the only substrates that were found to undergo enzymatic reduction to the hydroxylamines as a competing process. A mechanism was proposed that involves a redox reaction between the nitroso substrate and the enzymatic intermediate "active glycolaldehyde" at the active-site of transketolase.

The genotoxicity of aromatic amines and nitro compounds appears to be mediated by the production of one or more intermediate oxidation states. Metabolic oxidation of arylamines and partial reduction of nitro compounds to the arylhydroxylamine or nitroso metabolites are important bioactivation pathways for these chemicals [1, 2]. It appears that in some cases the production of the arylhydroxylamine metabolite is a sufficient activation reaction, since certain arylhydroxylamines can bind to macromolecules such as DNA without further metabolic activation [3–5]. In other cases, further bioactivation of the intermediate arylhydroxylamine is necessary, and O-acylation to produce a highly reactive acyloxyamine is considered to be an ultimate activation process [6–8]. Hydroxamic acids do require further activation such as O-sulfation [9], N-O acyltransfer or even simple deacylation [5] in order to be made sufficiently reactive towards macromolecules.

The status of the nitroso group as an ultimate genotoxicant is much less certain. The ease with which the nitroso functional group can be converted to the hydroxylamine oxidation state has prompted the suggestion that aromatic nitroso metabolites might be a source of ubiquitously-available activation products [10]. The relatively lipophilic nature of nitroso aromatics should facilitate their penetration of cell membranes [10]; therefore, intermediate nitroso metabolites could be important transport forms of N-oxidation products. In general, nitroso aromatics are reactive intermediates, and the prediction of their metabolic fate is not a simple process. The reactions of nitrosobenzene with certain thiamine-dependent enzymes have been reported previously [11–13]. The observation of the glycolic acid-derived hydroxamic acid following incubation of 4-chloronitrosobenzene with rat liver homogenates [12] has prompted us to consider the possibility that the enzyme transketolase might provide another general metabolic pathway, which nitroso metabolites can undergo. Accordingly, the purpose of this research was to examine the effects of ring substituents on the reaction of substituted nitroso aromatics with transketolase.

MATERIALS AND METHODS

Transketolase (Type X from Baker's Yeast), triosephosphate isomerase, α-glycerophosphate dehydrogenase, β-NADH (Grade III), thiamine pyrophosphate, D-fructose-6-phosphate (dipotassium salt), D-ribose-5-phosphate (disodium salt), D-xylulose-5-phosphate (sodium salt) and Tris · HCl buffers were purchased from the Sigma Chemical Co. Desferal mesylate was a gift from the Ciba-Geigy Co. All solvents were HPLC grade from Fisher Scientific. All other chemicals were of the highest purity available from commercial sources. High pressure liquid chromatography (HPLC) was done with a Waters Associates model 6000A pump, a model U6K septumless injector, a model 440 absorbance detector (dual wavelength) and a μBondapak C_{18} column (30 cm × 3.9 mm). Ultraviolet spectrophotometry was done with a Beckman model 35 spectrophotometer. NMR and MS analyses were obtained through the cooperation of the Department of Chemistry, Florida State University. Elemental analyses were conducted by Galbraith Laboratories.

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Preparation of substrates and metabolites. All the nitroso substrates have been reported previously in the chemical literature. Nitrosobenzene (4-H) itself was purchased from the Aldrich Chemical Co., and purified by co-distillation with 95% EtOH, followed by recrystallization from 95% EtOH. The 4-Cl, 4-CH_3, 4-OC_2H_5 and 4-C_6H_5 nitroso substrates were prepared by the use of the Zn-dust and NH_4Cl reduction of the corresponding nitro compounds [14, 15], followed by $FeCl_3$-oxidation of the intermediate arylhydroxylamine to the nitroso oxidation state [15]. The 4-NO_2, 4-CF_3 and 3-CF_3 nitroso substrates were prepared by oxidation of the corresponding arylamines with *m*-chloroperoxybenzoic acid according to a previously described procedure [16]. 4-Cl, 4-NO_2, 4-CF_3, 3-CF_3 and 4-CH_3 nitroso substrates were purified by distillation from aqueous EtOH followed by recrystallization from EtOH. 4-C_6H_5 and 4-OC_2H_5 nitroso substrates were purified by chromatography on EM-silica gel employing CH_2Cl_2/hexane mixtures as eluents, followed by recrystallization from EtOH.

Arylhydroxylamines were prepared either by Zn-dust reduction (4-H, 4-Cl, 4-CH_3, 4-OC_2H_5 and 4-C_6H_5) of the correspondi nitro aromatics (Aldrich Chemical Co.) or by ascorbic acid reduction (4-NO_2, 4-CF_3 and 3-CF_3) of the corresponding nitroso compounds [16, 17]. Recrystallization from Et_2O/hexane was used to obtain the purified arylhydroxylamines for use as HPLC standards.

The hydroxamic acids were prepared by the dicyclohexylcarbodiimide-driven acylation of the arylhydroxylamines with glycolic acid [11, 18]. Details for the synthesis of *N*-(4-biphenyl)glycolhydroxamic acid are given below. The preparation of the 4-H and 4-Cl hydroxamic acids has been reported previously [11, 18]. The NMR and MS properties of each hydroxamic acid were consistent with the assigned structures. Other physical characteristics of these compounds are the m.p. and elemental analysis, which are as follows (substituent, m.p., empirical formula and data from elemental analysis): 4-H (64.5–65.5°, $C_8H_9NO_3$, C 57.55, H 5.22, N 8.39); 4-Cl (124–125°, $C_8H_8NO_3Cl$, C 47.71, H 4.08, N 7.01); 4-CH_3 (119–119.5°, $C_9H_{11}NO_3$, C 59.72, H 6.00, N 7.56); 4-OC_2H_5 (121–122° decomp., $C_{10}H_{13}NO_4$, C 56.89, H 6.11, N 6.55); 4-NO_2 (187–188° decomp., $C_8H_8N_2O_5$, C 45.33, H 3.91, N 13.16); 4-CF_3 (112–114°, $C_9H_8NO_3F_3$, C 46.16, H 3.65, N 6.10); 3-CF_3 (90.5–91.5°, $C_9H_8NO_3F_3$, C 45.74, H 3.66, N 5.98).

Synthesis of N-(*4-biphenyl*)*glycolhydroxamic acid* [N,*2-dihydroxy*-N-(*4-biphenyl*)*acetamide*]. A suspension of 13 g (0.07 mole) of 4-hydroxylaminobiphenyl in 200 ml of anhydrous Et_2O contained in a 500-ml round bottomed flask was stirred by means of a magnetic stirbar, and cooled with an ice-water bath. To this stirred suspension was added 16.5 g (0.08 mole) of dicyclohexylcarbodiimide (Aldrich Chemical Co.) in 50 ml of Et_2O, followed by the addition over a 10-min period of 6.1 g (0.08 mole) of glycolic acid in 20 ml of dimethyl formamide. The ice bath was removed and stirring was continued for 40 min, at which time 200 ml of *n*-butanol was added, and stirring was continued for 15 min. The reaction mixture was filtered to remove dicyclohexylurea, and the filtrate was extracted twice with 100 ml of 0.8 N NaOH solution, then with 100 ml of H_2O. The combined aqueous extract was washed with 100 ml of Et_2O and then the pH adjusted to approximately 6 with 4 N HCl. The suspension was extracted with 100 ml of EtOAc, and the organic portion was washed with 50 ml of H_2O and 50 ml of NaCl-sat. H_2O. After drying (Na_2SO_4) and evaporation *in vacuo*, the resulting yellow solid was recrystallized from 50% EtOH to give 7.3 g (43%) of the desired product. Recrystallization from acetone/benzene/hexane (1:4:1) gave the highly-pure product as a pale yellow solid, m.p. 169° (decomp.); Analysis, calc. ($C_{14}H_{13}NO_3$): C 69.12, H 5.39, N 5.76; found: C 68.89, H 5.52, N 5.74; MS analysis: $(M)^-$ at m/z = 243, $(M\text{-}O)^+$ at m/z = 227; high resolution for m/z = 243 gave 243.0891 which is consistent for $C_{14}H_{13}NO_3$ (−0.4 millimass units (mmu) or 2 ppm difference from theory); NMR analysis, 200 MHz for ^1H (MeOH-D_4): δ 7.5 ppm (9H, mult.) δ 4.9 ppm (2H, singlet). UV analysis: λ_{max} (95% EtOH) = 280 nm (ε = 27,600).

Isolation and identification of product resulting from the action of transketolase on 4-*nitrosobiphenyl* (4-C_6H_5). To a solution of 95 ml of 0.05 M Tris·HCl, pH 7.4, at 37° was added fructose-6-phosphate (0.5 mmole) and a solution of 4-C_6H_5 nitroso substrate (1.8 mg, 0.01 mmole) in 400 μl of EtOH. The reaction was started by the addition of a solution of 1.4 mg of transketolase (25 μM units), thiamine pyrophosphate (0.01 mmole) and $MgCl_2$ (0.01 mmole) in 5 ml of 0.05 M Tris·HCl, pH 7.4. After incubation at 37° overnight, the reaction mixture was extracted twice with 50 ml of EtOAc, and the combined organic fraction was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was dissolved in 0.10 ml of MeOH, and the solution was diluted to 2.0 ml with CH_2Cl_2. The solution was chromatographed on a silica gel column (5 g of EM-silica gel 60 in a 1 × 12 cm bed volume) with 5% MeOH in CH_2Cl_2 as eluent. Fractions were monitored by HPLC analysis, and those fractions containing only the product were combined and evaporated. The residue was dissolved in 2.0 ml of EtOH, of which 0.10 ml was further diluted to 4.0 ml with EtOH. The remaining EtOH solution was evaporated to dryness and a portion was examined by MS analysis. The more dilute ethanolic solution was analyzed by u.v. spectrophotometry, which revealed the λ_{max} = 280 nm with A = 0.818 absorbance units. The u.v. spectra (200–350 nm) was identical to that of the synthetic standard of *N*-(4-biphenyl)glycolhydroxamic acid. Using ε = 27,600 (calculated from the u.v. spectrum of the authentic standard), the overall recovery of the pure hydroxamic acid metabolite was determined to be 24% based on the amount of the 4-C_6H_5 nitroso substrate employed in the enzymatic reaction.

Enzyme assay and incubation methods. Solutions of transketolase were prepared by dissolving 1–2 mg of the enzyme in 5.0 ml of 0.05 M Tris·HCl, pH 7.4 (at 25°), containing thiamine pyrophosphate and $MgCl_2$ at 2 mM each. After standing for at least 30 min at room temperature, portions of the enzyme solution were either used as such or further diluted with 0.05 M Tris·HCl, pH 7.4, prior to use. The

enzyme solutions were assayed daily by a standard technique [19], except that the temperature employed in the assay was 37° to correspond to the temperature employed in the incubations with nitroso substrates. A unit of transketolase activity is normally defined as that which produces 1 μmole of glyceraldehyde-3-phosphate per min from D-xylulose-5-phosphate at 25° in pH 7.7 glycylglycine buffer and in the presence of 5 mM D-ribose-5-phosphate. In this study, the activity was determined at 37° by the usual coupled enzyme assay in which the consumption of NADH is monitored at 340 nm. The enzymatic activity of transketolase solutions prior to any dilution was generally in the range of 12–28 units/mg of protein at 37°. Solutions of transketolase were stored at 4° and were used within 5 days after dissolution of the enzyme in buffer containing the cofactors at 2 mM.

Preliminary runs and time course studies were conducted in 10 cm × 2.2 cm (i.d.) Kimax test tubes sealed with silicon stoppers, and placed in a constant temperature water bath at 37°. For a typical time–course study, 9.0 ml of 0.05 M Tris·HCl, pH 7.4, containing 16.7 mg (50 μmoles) of D-fructose-6-phosphate was equilibrated to 37°; then the nitroso substrate was added as a solution in about 50 μl of EtOH, followed by the addition of 1.0 ml of enzyme solution containing from 1 to 6 units of transketolase activity to start the reaction. The test tube was stoppered, returned to the water bath, and then agitated briefly every 30 sec during the course of the reaction. At predetermined times, 1.0-ml aliquots were taken from the incubation and quenched by addition to 1.0 ml of MeOH cooled to −20°. The quenched aliquots were kept at −20° until HPLC analyses were done, which was generally within 2 hr.

A special methodology was employed for kinetic investigations in which losses of certain nitroso substrates due to volatilization had to be prevented. To a 1.0-ml Wheaton 400 serum vial (total capacity of 2 ml) was added 1.35 ml of 0.05 M Tris·HCl, pH 7.4, containing 2.5 mg (7.5 μmoles) of D-fructose-6-phosphate. The vial was sealed with a Wheaton aluminum seal (Teflon-faced syringe port), then equilibrated to 37° in a water bath. By means of a 25-μl syringe, the nitroso substrate (generally 0.075 μmole) was added to the sealed vial as a solution in 10 μl of EtOH just prior to the start of the reaction. To start the incubation, 150 μl of enzyme solution which contained from 1 to 6 units of transketolase activity per ml was added to the vial by means of a 250-μl syringe. After either a 1.0- or 2.0-min incubation period depending upon the substrate, a 50-μl aliquot was injected into the HPLC by means of a 100-μl HPLC syringe. Each study was conducted in quadruplicate, and all studies were repeated at least one time for each substrate.

RESULTS

In the presence of transketolase and an appropriate keto sugar such as fructose-6-phosphate, each of eight different aromatic nitroso compounds (Table 1) was found to be converted to the corresponding hydroxamic acid. Some of the nitroso substrates were also converted in part to the corresponding arylhydroxylamine. On the other hand, *N*,*N*-dimethyl-*p*-nitrosoaniline (0.1 mM) did not give any products detectable by HPLC upon reaction with transketolase and fructose-6-phosphate, even after a 1-hr incubation period. None of the nitroso substrates gave any detectable products in the absence of either fructose-6-phosphate or enzyme. The nature of the enzymatic products was inferred by use of HPLC. Retention times and optical absorbance ratios at two wavelengths for the products were compared to those determined for the authentic standards. The chromatographic properties of these products are listed in Table 1. The HPLC retention times and absorbance ratios of the products were shown to be identical to those of the standards by HPLC analysis of mixtures of the enzymatic products and standards. The authentic standards were synthesized by common methods, and their chemical structures were confirmed by NMR, MS and elemental analysis.

The preparation of hydroxamic acids based on glycolic acid as the acyl group has been reported [18]. The method involves N-acylation of the corresponding arylhydroxylamine with glycolic acid and dicyclohexylcarbodiimide. Details for the preparation of *N*-(4-biphenyl)glycolhydroxamic acid are in the experimental section. Similar procedures were used for the preparation of the other hydroxamic acid metabolites, although the glycolation of arylhydroxylamines with strong electron withdrawing substituents on the aromatic ring required more strenuous conditions in the form of longer reaction times and higher temperatures (20°). The glycolhydroxamic acids based on the 4-H and 4-Cl nitroso compounds have been prepared previously [11, 18], while the remaining six hydroxamic acids described in this study are new compounds.

As further proof of chemical structure of the hydroxamic acids, the product resulting from the action of transketolase on 4-nitrosobiphenyl was isolated from a large-scale enzymatic reaction. The MS of the metabolite was identical to that obtained for the synthetic standard, *N*-(4-biphenyl)glycolhydroxamic acid. In addition, the TLC properties of the isolated metabolite were identical to those of the synthetic standard; this included the development of a characteristic violet color of the hydroxamic acid zone on a TLC plate when sprayed with alcoholic $FeCl_3$ solution [20]. Confirmation of the arylhydroxylamine metabolites for those substrates that gave such a metabolite (see Table 2) was achieved by treatment of a quenched aliquot of the enzymatic reaction with 1% $FeCl_3$ in methanol. This resulted in the oxidation of the arylhydroxylamine to the corresponding nitroso compound, and this conversion was then detected by HPLC analysis.

HPLC analysis of the enzymatic reaction mixtures was designed to provide quantitative determinations of both products and starting material. Optimal peak symmetry for the hydroxamic acid products was achieved by use of desferal mesylate as a solvent additive [21]. It has been reported that desferal mesylate will also decrease the oxidation of arylhydroxylamine metabolites during HPLC analysis [22]. Figure 1 illustrates representative chromatograms that were generated by direct HPLC analysis of reaction aliquots or MeOH-quenched aliquots. Sol-

Table 1. Chromatographic properties of nitroso substrates and transketolase-generated metabolites*

Compound†	40% MeOH R.T. (min)	40% MeOH $\frac{\lambda 254}{\lambda 313}$	50% MeOH R.T. (min)	50% MeOH $\frac{\lambda 254}{\lambda 313}$	Other‡ R.T. (min)	Other‡ $\frac{\lambda 254}{\lambda 313}$
4-NO_2 Nitroso	9.1	0.8	—	—	—	—
4-NO_2 H.A.	5.1	0.4	—	—	—	—
4-NO_2 NHOH	3.7	0.5	—	—	—	—
4-CF_3 Nitroso	—	—	14.4	1.2	—	—
4-CF_3 H.A.	—	—	6.7	13.0	—	—
4-CF_3 NHOH	—	—	5.5	ND§	—	—
3-CF_3 Nitroso	—	—	15.4	1.2	—	—
3-CF_3 H.A.	—	—	5.9	ND	—	—
3-CF_3 NHOH	—	—	5.1	ND	—	—
4-H Nitroso	—	—	—	—	18.3	0.33
4-H H.A.	—	—	—	—	4.7	16.7
4-Cl Nitroso	—	—	10.8	0.2	—	—
4-Cl H.A.	—	—	4.3	16.3	—	—
4-CH_3 Nitroso	17.9	0.1	—	—	—	—
4-CH_3 H.A.	4.7	14.3	—	—	—	—
4-C_6H_5 Nitroso	—	—	—	—	14.6	1.3
4-C_6H_5 H.A.	—	—	—	—	4.9	8.0
4-OC_2H_5 Nitroso	21.7	0.4	10.8	0.4	—	—
4-OC_2H_5 H.A.	4.8	10.8	3.4	11.5	—	—

* All solvents also contained 0.01% desferal mesylate and were buffered with 0.01 M KH_2PO_4 buffer, pH 3.5. Solvent flow rate was 1.5 ml/min through a μBondapak C_{18} column (30 cm × 3.9 mm, i.d.).

† The nomenclature was generated by indicating the nature of the substituent group and its position on the benzene ring relative to the basic functional groups, which are the nitroso, the *N*-glycolyl hydroxamic acid (H.A.) and the hydroxylamine (NHOH) functional groups.

‡ The other solvent was 30% MeOH for the chromatographic analysis of the 4-H series and 60% MeOH for the analysis of the 4-C_6H_5 series, both of which also contained desferal mesylate and buffer.

§ ND indicates that the wavelength ratio was not calculated since the three compounds in question displayed little or no absorbance at λ313.

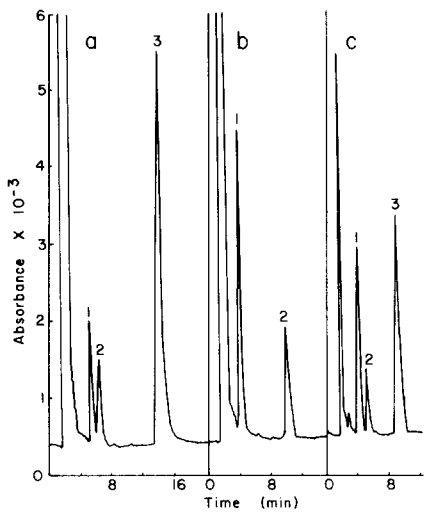

Fig. 1. High pressure liquid chromatograms of aliquots from incubations of nitroso substrates with transketolase. The chromatographic conditions are described in Table 1. The chromatograms are illustrative for three different nitroso substrates. Chromatograms a and b were obtained at 254 nm; chromatogram c was obtained at 313 nm. Peak identification and the amount of compound which produced each peak is as follows: (a) 4-CF_3 nitroso (3, 529 ng), hydroxylamine (1, 37 ng), hydroxamic acid (2, 16 ng); (b) 4-Cl nitroso (2, 313 ng), hydroxamic acid (1, 36 ng); and (c) 4-NO_2 nitroso (3, 330 ng), hydroxylamine (1, 71 ng), hydroxamic acid (2, 23 ng).

vents were selected to provide sufficient resolution of the hydroxamic acid and arylhydroxylamine metabolites, yet still allow for a reasonably short retention time for the nitroso substrates, which are much more lipophilic (Table 1). Such HPLC methods allowed for the determination of a material balance, which proved to be quite useful.

Only the nitroso substrates with the nitro and trifluoromethyl groups (4-NO_2, 3-CF_3 and 4-CF_3) gave the arylhydroxylamine metabolites in addition to the glycolhydroxamic acid products (see Table 2). No other metabolites were detected by HPLC for any of the substrates, except under certain conditions where the nitro and trifluoromethyl substituted nitroso substrates were found to produce a small amount of the azoxy products. The azoxy products were identified by HPLC retention times employing 80% MeOH as the solvent (data not shown). Presumably, the azoxy compounds result from condensation of the arylhydroxylamine metabolites with unreacted substrate. The presence of the azoxy compound was observed only in those incubation reactions that generated the hydroxylamine, and that were conducted for more than about 5 min or in quenched aliquots after several hours. The production of the azoxy metabolites was negligible in all situations where quantitative rate data was generated.

The substitution of the corresponding arylhydroxylamines for certain of the nitroso compounds

(4-NO_2, 4-Cl and 4-CH_3) in the enzymatic reactions with transketolase and fructose-6-phosphate failed to result in the production of any hydroxamic acid product. The incubation of the hydroxamic acids derived from the 4-NO_2, 4-CF_3 and 3-CF_3 nitroso substrates with transketolase under the usual conditions failed to produce any of the corresponding arylhydroxylamine or nitroso aromatic. This proved that the production of arylhydroxylamines in the cases of 4-NO_2, 4-CF_3 and 3-CF_3 nitroso substrates was not due to hydrolysis of the hydroxamic acid metabolites.

For the nitroso substrates that possessed strong electron withdrawing substituents (4-NO_2, 4-CF_3 and 3-CF_3), the possibility that arylhydroxylamine production during the course of the enzymatic reaction might be due to simple chemical reduction of these substrates was investigated. These three nitroso substrates (50 μM) were found to be stable during incubations with fructose-6-phosphate and cofactors. Also, the addition of ribose-5-phosphate (0.5 mM) to these controls failed to result in the production of any of the arylhydroxylamines or other detectable products for at least 30 min. This demonstrated that simple chemical reduction of the nitroso substrates by an aldose sugar is not a significant reaction under the conditions of the enzymatic reactions.

Progress curves for the transketolase-catalyzed conversions of representative nitroso substrates to the hydroxamic acids are illustrated in Figs. 2 and 3. The 4-C_6H_5 nitroso substrate gave only the hydroxamic acid product during the course of the incubation, and the rate of this conversion was relatively slow (Fig. 2). On the other hand, the 4-NO_2 nitroso substrate was rapidly converted to two metabolites, which were the hydroxamic acid and the arylhydroxylamine (Fig. 3). In a non-enzymatic reaction, the azoxy compound was also produced (Fig. 3). Quantitative determination of the azoxy product was achieved by HPLC analysis employing 80% MeOH in a separate run. A characteristic of most of the progress curves was the lack of a material

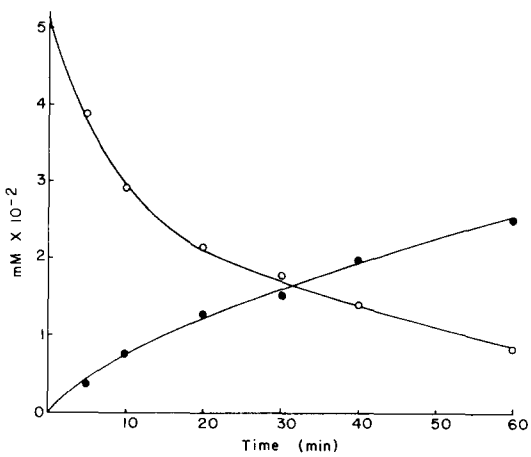

Fig. 2. Progress curve for the action of transketolase on the 4-C_6H_5 nitroso substrate. The amount of 4-C_6H_5 nitroso substrate (○) and its hydroxamic acid metabolite (●) that was present in an incubation containing the substrate at an initial concentration of 0.05 mM with transketolase at 28 μg/ml (0.45 units/ml) is given as a function of time.

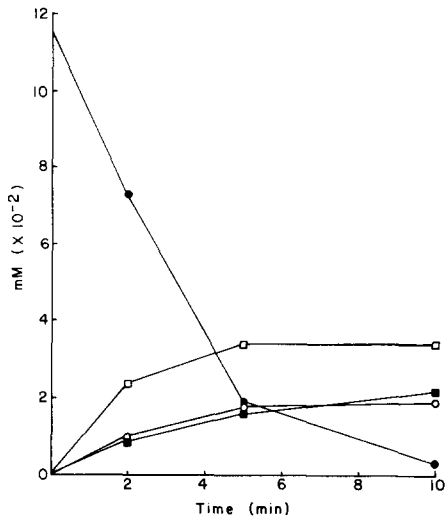

Fig. 3. Progress curve for the action of transketolase on the 4-NO_2 nitroso substrate. The amount of 4-NO_2 nitroso substrate (●), 4-NO_2 hydroxamic acid (■), 4-NO_2 hydroxylamine (○) and azoxy compound (□) that was present in an incubation containing the substrate at an initial concentration of 0.12 mM with transketolase at 14 μg/ml (0.31 units/ml) is given as a function of time.

balance, which became especially obvious after about 5 min reaction time, depending upon the substrate. The HPLC methodology was particularly suited to provide such information since the substrate and all initial products are determined simultaneously. The major cause of the observed material imbalance was due to the rather significant vapor pressure of most of the aromatic nitroso compounds at 37°. The volatility was greatest for the two trifluoromethyl-substituted nitroso compounds, and a special technique was developed to minimize the loss of these and the other substrates during their reaction with transketolase. Tight sealing of the reaction vessels and the near-elimination of all head space in the reaction vessels was necessary to minimize losses due to evaporation of the nitroso substrates. Such losses would cause appreciable errors in the determination of initial-rate data even for aliquots that were analyzed after only a 1-min reaction period. In the case of the 4-C_6H_5 nitroso substrate, significant losses arise from the coating-out of this substrate onto the surfaces of the reaction vessel.

It was originally planned to determine the kinetic constants, K_m and V_{max}, for the reaction of transketolase and fructose-6-phosphate with each of the eight nitroso aromatic substrates. The usual approach of investigating the initial rate of reaction (<5% substrate consumption) as a function of nitroso substrate concentration was not successful for several possible reasons. The 4-C_6H_5 nitroso substrate is not sufficiently soluble in aqueous systems to allow for a study of the effect of concentration upon the rate of the reaction at concentrations in excess of 0.2 mM. The results of experiments with various concentrations of substrate conducted with the 4-Cl, 4-OC_2H_5 and 4-CH_3 nitroso substrates suggested that these enzymatic reactions do not appear

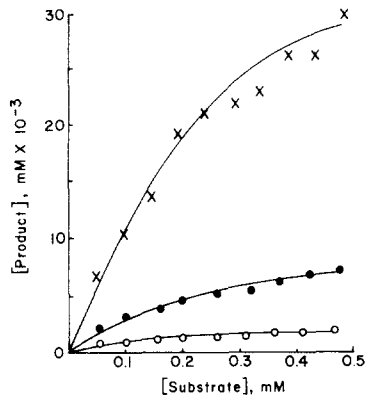

Fig. 4. Effect of substrate concentration on the initial rates of reaction of selected nitroso substrates with transketolase. The concentrations of the hydroxamic acid metabolites at T = 1.0 min were plotted as a function of the initial substrate concentrations for the 4-Cl nitroso (×), 4-CH_3 nitroso (●) and 4-OC_2H_5 nitroso (○) substrates. The concentration of enzyme was 0.028 mg/ml for each of the three substrates; however, the total transketolase activity present was 0.67 units/ml (4-Cl), 0.52 units/ml (4-CH_3), and 0.45 units/ml (4-OC_2H_5).

to obey the simple Michaelis equation, even though plots of V_i vs [S] gave rectangular hyperbolas (Fig. 4) [23]. Yeast transketolase possesses two catalytic centers that differ with respect to their affinity for the cofactors and with respect to their inactivation by irreversible inhibitors [24]. Such a feature of the enzyme is expected to complicate kinetic analyses. Graphical analysis by Lineweaver–Burk and Woolf–Hofstee plots [23] of the initial-rate data from these concentration-dependency studies suggested that enzyme saturation was not achieved within the solubility range of the nitroso substrates. Data generated at concentrations below the solubility limit of the substrates gave K_m and V_{max} values that varied significantly with the actual substrate concentration ranges that were employed in these studies (data not shown). Thus, the calculated values for K_m and V_{max}/K_m (Table 2) are only considered to be approximations. It was decided to obtain the kinetic constant K_{cat} for the eight substrates through an analysis of the enzymatic reactions at low substrate concentrations, where first-order kinetics are being followed. K_{cat} is the rate constant for the apparently first-order region of the V_i vs [S] curve, and is equivalent to the ratio V_{max}/K_m [23]. The K_{cat} values for the eight substrates were determined as the rate constants for the reactions when the initial substrate concentrations employed were about 50 μM. This concentration was selected on the basis of being in or at least near to the first-order region of the reaction for all the substrates employed, yet was a concentration that allowed for sufficient analytical sensitivity without resulting in the consumption of more than 5% of the initial substrate at the sampling time. Special conditions were employed to prevent any significant loss due to volatility of the substrates during the 1.0-min reaction times. In the absence of such methodology, losses due to volatilization in systems with relatively large head-spaces (e.g. >0.5 vol.) were as

Table 2. Kinetic constants for the transketolase-catalyzed conversions of nitroso aromatic substrates*

Substrate	V_{max}/K_m† (ml/min·U)	K_{NHOH}‡ (ml/min·U)	K_{HA}‡ (ml/min·U)	K_{cat}‡§ (ml/min·U)	$E_{0.5}$‖ (mV)
4-NO_2	—	0.63	0.14	0.77 ± 0.060	−430
4-CF_3	—	0.15	0.05	0.20 ± 0.016	—
3-CF_3	—	0.08	0.07	0.16 ± 0.013	—
4-H	—	0	0.15	0.15 ± 0.009	−479
4-Cl	0.16	0	0.11	0.11 ± 0.008	−469
4-CH_3	0.07	0	0.05	0.05 ± 0.004	−525
4-C_6H_5	—	0	0.05	0.05 ± 0.003	—
4-OC_2H_5	0.04	0	0.02	0.02 ± 0.002	−598¶

* The rate constants are reported as second-order rate constants with the enzyme concentration expressed on the basis of μM units (U) of transketolase.

† The calculation of V_{max}/K_m values was made from studies on the initial rates of the reactions as functions of initial substrate concentrations. Both Lineweaver–Burk and Woolf–Hofstee plots [23] were employed in the analysis of two or more independent studies. The values reported are from a single study in which both graphical methods gave similar values for K_m. The K_m values were found to be 0.34, 0.24 and 0.14 μmole/ml for the 4-Cl, 4-CH_3 and 4-OC_2H_5 nitroso substrates respectively.

‡ K_{NHOH} and K_{HA} are the individual rate constants for the transketolase-catalyzed conversions of the nitroso substrates to the arylhydroxylamine and hydroxamic acid metabolites respectively. K_{cat} is the sum of these two rate constants in the cases of the 4-NO_2, 4-CF_3 and 3-CF_3 substrates; otherwise, K_{cat} is identical to K_{HA}.

§ The precision of the values for K_{cat} was calculated as the standard deviation determined from quadruplicate runs. The major contribution to the error originated from the low precision of the assay for enzyme activity.

‖ Reduction potentials were taken from the literature [25], and are relative to a saturated Hg_2SO_4 electrode.

¶ Literature value [25] was reported for the 4-OCH_3 analog, which should be close to that expected for 4-OC_2H_5.

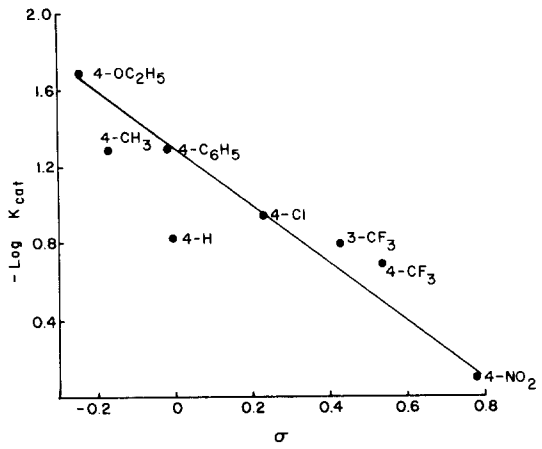

Fig. 5. Plot of $-\log K_{cat}$ versus Hammet substituent constants (σ).

high as 20% within 1 min for the trifluoromethyl substrates; and such loss of substrate would contribute to a rather significant error in K_{cat}. The values of K_{cat} (Table 2) for those substrates that were converted in part to the arylhydroxylamine metabolite are expressed as the sum of the individual rate constants (K_{NHOH} and K_{HA}) for the production of the arylhydroxylamine and the hydroxamic acid products respectively. Even though the experimental methods used for the determination of the mathematically-equivalent constants K_{cat} and V_{max}/K_m were quite different, these values for the 4-Cl, 4-CH_3 and 4-OC_2H_5 nitroso substrates are similar (Table 2).

DISCUSSION

The transketolase-catalyzed conversion of nitroso aromatic substrates to N-glycolyl derived hydroxamic acids appears to be a fairly general reaction for the nitroso functional group. Of nine C-nitroso substrates investigated, all but one displayed this reactivity with transketolase. Even the unreactive substrate, *N,N*-dimethyl-*p*-nitrosoaniline, fits the observed pattern of substituent effects although to an extreme degree, in that electron-donating functional groups were found to retard the rate of the reaction. For certain nitroso substrates, the production of an appreciable amount of the arylhydroxylamine metabolite was found to occur simultaneously with hydroxamic acid production. For the case of 4-NO_2 and 4-CF_3 nitroso substrates, the rate of production of the hydroxylamine metabolites actually exceeded the rates for hydroxamic acid production. The effect of substituents upon the combined rates (K_{cat}) of production of the arylhydroxylamine and hydroxamic acid metabolites is illustrated in Fig. 5, in which the plot of $-\log K_{cat}$ versus Hammet substituent constants (σ) shows a fairly good linear correlation (Fig. 5). The rates of the reactions (K_{cat}) increased as the electron-withdrawing properties of the substituent increased.

The production of arylhydroxylamines from three of the nitroso substrates appears to require the presence of a strong electron-withdrawing substituent on the aromatic ring. The rate of production of the corresponding arylhydroxylamine was found to increase as the effective electron-withdrawing power of the substituent increased. Thus, the electron-withdrawing properties of the substituent group affect both the rate and the nature of the products of the transketolase-catalyzed reaction. It was not feasible to determine the least electronegative substituent group that would cause the production of the arylhydroxylamine metabolite at a detectable level, but it would be expected to be a substituent that is between 3-CF_3 and 4-Cl in electronegativity.

The relative rates of reaction of the various nitroso substrates with transketolase were found to increase as the reduction potentials increased within the series of substrates (Table 2). This correlation is expected since all reasonable mechanisms to explain the reaction between the nitroso functional group and the carbanion-like species at the active-site of transketolase ("active glycolaldehyde") involve either electron transfer from the active-site species to the nitroso functional group (Fig. 6), or nucleophilic attack of the active-site species on the N atom of the nitroso group [13]. In several studies, it has been found that the reduction potential for the conversion of the nitroso functional group to the arylhydroxylamine increased as the electron-withdrawing

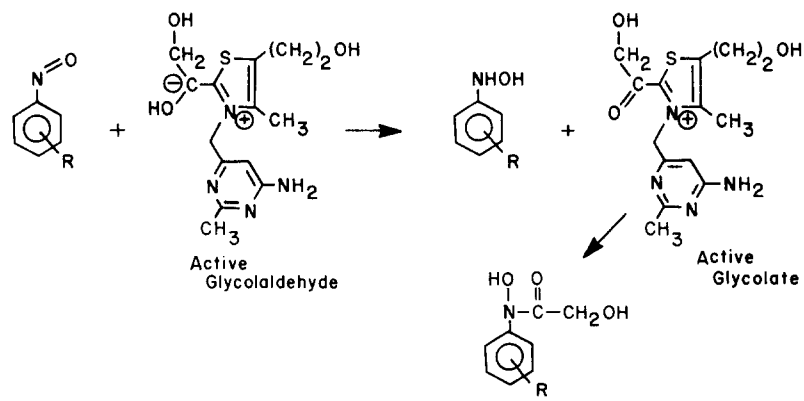

Fig. 6. Proposed mechanism for the transketolase-catalyzed conversion of nitroso aromatics to hydroxamic acids. Thiamine pyrophosphate and the C_2-adducts of thiamine pyrophosphate are bound at the active-site of the enzyme.

ability of substituent groups increased [15, 25]. Thus, the redox mechanism (Fig. 6) explains the observed effect of substituents upon the rate of reaction in this transketolase reaction.

The chemical reaction of nitroso aromatics with thiols such as glutathione has been elucidated [26–31], and this reaction is thought to be a major route for the detoxification of nitroso aromatic metabolites. Although this reaction with glutathione is rather complex, it is known that the nature of the substituent on the aromatic ring of the nitroso substrate has a profound effect on the rate and nature of products produced [30]. The reaction with glutathione proceeds by an initial rapid and reversible adduct formation with the nitroso substrate [30]. As would be expected for a nucleophilic attack upon the nitroso N atom, this initial reaction has been found to proceed more rapidly when electron-withdrawing substituents are present on the aromatic ring of the nitroso compound [30]. This effect of structure on reactivity displays the same trend as that observed for the reaction of nitroso aromatics with transketolase; however, the reaction with glutathione appears to be even more sensitive to the electronic effect of substituents (e.g. a 470-fold rate increase in going from 4-OC_2H_5 to 4-nitrosoacetophenone in the glutathione reaction versus a 40-fold rate increase in going from 4-OC_2H_5 to 4-NO_2 in the present study).

Although the redox mechanism illustrated in Fig. 6 can explain all the observations made for the action of transketolase on nitroso substrates, it is not possible at this time to exclude nucleophilic attack of the active-site carbanion on the nitroso substrates as a competing process, at least with respect to the production of the hydroxamic acid metabolites. On the other hand, a nucleophilic mechanism alone does not readily explain the production of the hydroxylamine metabolites that were observed for some of the nitroso substrates.

Except for the nature of the C_2 adduct with thiamine and, thus, the structure of the hydroxamic acid product, the redox mechanism in Fig. 6 is identical to the mechanism proposed for the action of pyruvic acid decarboxylase on nitroso aromatics [13]. In that reaction, the production of considerable arylhydroxylamine accompanied the production of the acetyl-derived hydroxamic acid [13]. Even 4-chloronitrosobenzene (4-Cl) was converted in large part to 4-chlorophenylhydroxylamine, a process which was not observed in the transketolase reaction.

A $2e^-$ transfer from the "active glycolaldehyde" to the nitroso substrate is probably the rate-limiting step for the transketolase-catalyzed reaction. Thus, those nitroso substrates that are more easily reduced give higher observed rate constants. This initial redox reaction produces the arylhydroxylamine which is bound at enzyme active site, and most likely in close proximity to the "active glycolate". The "active glycolate" intermediate is an acylthiazolium species and is probably a reactive species [32] that can acylate the bound arylhydroxylamine. Arylhydroxylamines are generally strong nucleophiles with the N atom being the center for this nucleophilicity [33]. Therefore, in most cases, the "active glycolate" and arylhydroxylamine rapidly condense to give the hydroxamic acid, which then diffuses from the enzyme active site. Exceptions arise for those cases in which the bound arylhydroxylamine is a relatively weak nucleophile. Although we do not have any literature values with which to express and compare the relative nucleophilicities of arylhydroxylamines, it is to be expected that as arylhydroxylamines are substituted on the ring with stronger electron-withdrawing groups, the nucleophilic character of the hydroxylamine N atom will decrease proportionately. For arylhydroxylamines possessing substituents such as the nitro and trifluoromethyl groups, the condensation with "active glycolate" at the enzyme active site is relatively slow. If, as proposed, the rate-determining step is the initial redox step, then the rate of this condensation reaction will have no effect upon the kinetics of the overall reaction, even for nitroso aromatics possessing the nitro or similar strong electron-withdrawing groups. On the other hand, the sluggishness of the condensation reaction may allow sufficient time for the bound arylhydroxylamine to escape from the enzyme active site. Thus, the hydroxylamine becomes a detectable metabolite for the transketolase-catalyzed process only when the parent substrate possesses sufficiently strong electron-withdrawing groups.

Transketolase from yeast was employed in this study because mammalian transketolases are typically more unstable [34]. There is no reason to expect any major differences in reaction mechanisms among the known transketolases; therefore, results obtained with yeast transketolase should be generally applicable to other organisms, or at least serve as a basis for comparison. The partial conversion of 4-chloronitrosobenzene to the glycolhydroxamic acid (4-Cl) by rat liver homogenates [12] and nitrosobenzene to the glycolhydroxamic acid (4-H) by a unicellular algal species [35] tend to support this proposal. More recently, the conversions of several nitroso aromatic substrates to the glycolhydroxamic acids have been found to occur to the extent of several percent in isolated rat liver hepatocytes (M. D. Corbett, unpublished results). Thus, the glycolhydroxamic acids are potential minor metabolites of nitroso aromatics in a large number of tissues and organisms. The transketolase-catalyzed reduction of certain nitroso aromatics to their arylhydroxylamines is another metabolic reaction that may contribute to the *in vivo* production of arylhydroxylamines in a manner analogous to that of glutathione and other biological reductants. The extent of these conversions and their significance to the toxic actions of such chemicals are being investigated.

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