BIOORGANIC CHEMISTRY 12, 206–220 (1984)

Functionalized Cyclodextrins as Holoenzyme Mimics of Thiamine-Dependent Enzymes

DONALD HILVERT AND RONALD BRESLOW*

Department of Chemistry, Columbia University, New York, New York 10027

Received January 28, 1984

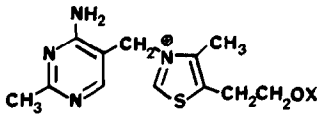
Mimics of vitamin B_1-dependent enzymes have been prepared by covalently linking thiazolium salts to the primary side of β-cyclodextrin. Although these compounds were found to catalyze the benzoin condensation, they were generally less effective turnover catalysts than the analogous simple thiazolium salts lacking the artificial binding site. An improvement in the design of the sugar derivative which prevented competitive binding of the thiazolium moiety to the cyclodextrin cavity did, however, lead to a superior catalyst of this transformation. In catalytic processes which required only *one* molecule of substrate to react with the thiazolium salt, rate accelerations, saturation kinetics, and substrate selectivity were observed. Thus, all the cyclodextrin thiazolium salts speeded the rate of tritium exchange from suitably labeled aromatic aldehydes more than did simple thiazolium salts. In addition, rate enhancements of up to ca 40-fold were observed for the thiazolium-catalyzed oxidation of *tert*-butylbenzaldehyde by ferricyanide compared with a thiazolium salt lacking the cyclodextrin binding group.

INTRODUCTION

The development of simple chemical models of enzyme action is currently of considerable interest (*1*). In addition to providing a deeper understanding of the chemical underpinnings of biological catalysis, enzyme mimics may find far-reaching application in medicine and industry as viable catalytic species in their own right. For some time now we have been interested in the use of functionalized cyclodextrins as practical models of the active site of enzymes, and have recently reported the preparation and properties of artificial "transaminases" (*2*, *3*). The coenzyme pyridoxamine was selectively attached to both the primary (C-6) and secondary (C-3) faces of β-cyclodextrin, and the resulting derivatives were shown to facilitate the conversion of α-ketoacids into α-aminoacids. The finding that rate enhancements and optical induction were attained in the reactions of these cyclodextrin compounds with substrates containing aromatic side chains suggests a significant degree of cooperative catalysis between coenzyme and binding site.

Thiamine pyrophosphate **1** is an essential cofactor for numerous enzymes which promote the formation and cleavage of carbon–carbon bonds. This important coenzyme facilitates the generation of acyl anion synthons (*4*), and is responsible for *in vivo* catalysis of such reactions as the decarboxylation of α-ketoacids, acyloin condensations, and transketolizations. Furthermore, thiamine and simple

0045-2068/84 $3.00



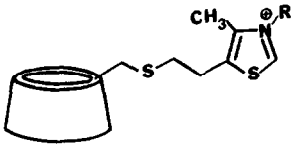
1a X=H

1b X=$P_2O_6^{3-}$

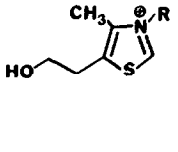
thiazolium salts have been shown to catalyze many of these transformations in the absence of enzymes, albeit with significantly reduced rates and selectivities (*4*). Given the importance of proximity and orientation factors for effective catalysis, we decided to link thiamine and simple thiazolium analogs to β-cyclodextrin in analogy to our work with pyridoxamine.

EXPERIMENTAL PROCEDURES

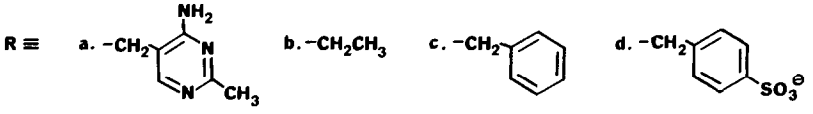
Chemicals were purchased from Aldrich unless otherwise indicated. Thiamine hydrochloride was obtained from Hoffmann–La Roche, Inc. Tritiated sodium borohydride (NaB^3H_4) was purchased from Amersham. Dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were dried by stirring over calcium hydride overnight and distilling. 3-Ethyl-4-methyl-5-(2-hydroxyethyl)thiazolium bromide **3b** (*5*) and 3-benzyl-4-methyl-5-(2-hydroxyethyl)thiazolium chloride **3c** (*6*) were prepared according to literature methods.



2



3



4 - Methyl - 5 - [2 - S - (β - cyclodextrin - 6^A - deoxy - 6^A - thio)ethyl]thiazole (*β-cyclodextrin thiazole*) **4.** A solution of 4-methyl-5-(2-mercaptoethyl)thiazole (*7*) (1.22 g, 7.66 mmol) in 3 ml DMF was added to a solution of β-cyclodextrin-6-monotosylate (*8*) (3.29 g, 2.55 mmol) in 100 ml water at 65°C, and the reaction was allowed to stir overnight under nitrogen at that temperature. After cooling to room temperature, the product was precipitated by addition of 400 ml acetone, collected by filtration, and washed successively with acetone and then ether. The crude product (3.03 g, 93%) was contaminated by both starting materials, as seen by

TLC. 4-Methyl-5-(2-mercaptoethyl)thiazole was removed by redissolving the crude material in aqueous DMF and extracting 8–10 times with ether. The aqueous layer was then concentrated *in vacuo,* and the dry residue was recrystallized from the minimum amount of water. The isolated product showed no detectable contamination by TLC or NMR. R_f 0.45 [silica gel, 5 : 7 : 7 (v/v/v) H_2O : EtOAc : i-PrOH]; MS (CI-NH_3): *m/e* 1274 (M-1); uv (H_2O): λ_{max} 252 nm; ^1H NMR (250 MHz, DMSO-d_6) δ: 8.78 (s, 1H), 5.92–5.65 (bm, 14H), 4.88–4.78 (bm, 7H), 4.57–4.44 (bm, 6H), 3.83–3.21 (bm, not integrated), 2.97 (t, J = 7 Hz, 2H), 2.75 (t, J = 7 Hz, 2H), 2.28 (t, 3H).

3 - [(2' - Methyl - 4' - amino - 5' - pyrimidyl)methyl] - 4 - methyl - 5 - [2 - S - (β - cyclodextrin - 6^A - deoxy - 6^A - thio)ethyl]thiazolium chloride, **2a**. Cyclodextrin thiazole **4** (250 mg, 0.196 mmol), 2-methyl-4-amino-5-(bromomethyl)pyrimidine (*9*) (48 mg, 0.239 mmol), and a catalytic amount of potassium iodide (4.0 mg, 0.024 mmol) were dissolved in 2.5 ml dry DMF under argon, and the resulting solution was stirred at 90°C. Two additional 50-mg portions of alkylating agent were added after 3 and 6 hr, respectively. After 11.5 hr at 90°C, the reaction was cooled and allowed to stand overnight at room temperature. A yellowish solid precipitated from solution upon addition of acetone. The latter was collected by filtration, redissolved in 1 ml water, and precipitated again with acetone. The crude product (282 mg, 97%) was isolated by filtration, washed with acetone, dried *in vacuo,* and purified in two 140-mg batches by chromatography on a Sephadex CM-25 column (3.25 × 22.5 cm). The material was dissolved in 140 ml water, loaded onto the column, and eluted with a continuous gradient of 0.0 M (1.8 liters)–0.2 M (1.8 liters) NH_4HCO_3 (pH 7.9). The thiazolium derivative typically eluted at 1100–1400 ml. After the water and most of the buffer had been removed by lyophilization, the residue was dissolved in 1 ml deionized water and chromatographed on 8 g Bio-Rad anion-exchange resin AG3-X4A (chloride form, weakly basic). The uv-active fractions were combined and lyophilized. The total yield of **2a**, as the chloride salt, was 113 mg (39%). Secondary ion mass spectrum (10): *m/e* 1397 (M + H − Cl), 1489 (M + glycerol + H − Cl); uv (H_2O): λ_{max} 269, 283 nm; ^1H NMR (250 MHz, acidic D_2O) δ: 9.54 (s, 1H, exchanged at neutral pH); 7.90 (s, 1H); 5.44 (s, 2H); 4.95 (bs, 7H); 4.01–3.37 (bm, not integrated); 3.19 (t, J = 6.6 Hz, 2H); 2.90 (t, J = 6.6 Hz, 2H); 2.52 (s, 3H); 2.44 (s, 3H).

3 - Ethyl - 4 - methyl - 5 - [2 - S - (β - cyclodextrin - 6^A - deoxy - 6^A - thio)ethyl] thiazolium chloride, **2b**. Cyclodextrin thiazole **4** (510 mg, 0.40 mmol) was dissolved in 15 ml dry DMF in a flask equipped with a reflux condenser. Ethyl bromide (1.19 ml, 16 mmol) in 10 ml acetonitrile was added, and the resulting solution was refluxed under nitrogen. After 12 hr, additional ethyl bromide (0.43 ml) was added and the reflux was continued overnight (18 hr). The DMF solution was then concentrated *in vacuo,* and the product was precipitated from solution by the addition of acetone, collected by filtration, washed with ether, and dried under vacuum. The crude product was chromatographed in 150 to 200-mg batches on a Sephadex CM-25 column (ca. 3.5 × 30 cm). The material was dissolved in 150–200 ml water and eluted with a continuous gradient of 0.0 M (1.6 liters)–0.2 M (1.6 liters) NH_4HCO_3 (pH 7.9). The thiazolium cyclodextrin typically eluted at 900–1400 ml. After the water and most of the buffer had been removed

by lyophilization, the residue was dissolved in 1 ml deionized water and chromatographed on 8 g Bio-Rad anion exchange resin AG3-X4A (chloride form, weakly basic). A combined yield of 241 mg (44%) of **2b** was obtained as the chloride salt after lyophilization of all the batches. UV (H_2O): λ_{max} 259.2 nm; ^{1}H–NMR (250 MHz, acidic D_2O) δ: 9.53 (s, 1H); 5.20 (bs, 2H); 4.87 (bs, 5H); 4.24 (q, J = 8 Hz, 2H); 4.0–3.3 (m's, not integrated); 3.04 (bt, 2H); 2.78 (bm, 2H); 2.31 (s, 3H); 1.35 (t, J = 8 Hz, 3H).

3 - Benzyl - 4 - methyl - 5 - [2 - S - (β - cyclodextrin - 6^A - deoxy - 6^A - thio)ethyl]thiazolium chloride, **2c**. Cyclodextrin thiazole **4** (638 mg, 0.5 mmol) and a catalytic amount of potassium iodide (8.3 mg, 0.05 mmol) were dissolved in 5 ml dry DMF under argon. Benzyl bromide (71.4 μl, 0.6 mmol) was added dropwise, and the resulting solution was stirred at 85–90°C. Two additional portions of benzyl bromide (36 μl, 0.3 mmol) were added after 5 and 9 hr, respectively. After a total of 11 hr at 85–90°C, the reaction was cooled to room temperature, and the solvent was removed *in vacuo*. The residue was taken up in 5 ml water and washed four times with 4 ml ether. The volume of the aqueous layer was then reduced *in vacuo* to 2 ml, and the crude product was precipitated by the addition of acetone, collected by filtration, washed with acetone and ether, and dried *in vacuo*. The 558 mg (79%) of material thus obtained was chromatographed in 150-mg batches on a Sephadex CM-25 column (ca. 3.5 × 30 cm). The material was dissolved in 150 ml water and eluted with a continuous gradient of 0.0 M (1.6 liters)–0.2 M (1.6 liters) NH_4HCO_3 (pH 7.9). The desired product typically eluted at 1100–1600 ml. After the water and most of the buffer had been removed by lyophilization, the residue was dissolved in 1 ml deionized water and chromatographed on 8 g Bio-Rad anion exchange resin AG3-X4A (chloride form, weakly basic). Lyophilization of the uv-active fraction from a typical batch yielded 101 mg of the *N*-benzyl thiazolium cyclodextrin **2b** which was pure by TLC and ^{1}H NMR. UV (H_2O): λ_{max} 259.2 nm; ^{1}H NMR (250 MHz, acidic D_2O) δ: 9.76 (s, 1H); 7.39–6.93 (m's, 5H); 5.54 (ABq, J = 9.3, 14.4 Hz, 2H); 4.84 (bm, 7H); 3.9–3.3 (m's, not integrated); 3.2–2.5 (m's, not integrated); 2.15 (s, 3H).

3 - (4 - Sulfobenzenemethyl) - 4 - methyl - 5 - [2 - S - (β - cyclodextrin - 6^A - deoxy - 6^A - thio)ethyl]thiazolium, **2d**. Cyclodextrin thiazole **4** (500 mg, 0.392 mmol), sodium 4-(bromomethyl)benzenesulfonate (11) (60 mg, 0.239 mmol), and a catalytic amount of potassium iodide (7.5 mg, 0.045 mmol) were dissolved in 5 ml dry DMF under argon, and the resulting solution was stirred at 95°C. Seven additional 60-mg portions of alkylating agent were added after 0.5, 3.0, 3.5, 6.0, 6.5, 10.0, and 10.5 hr. After a total of 12 hr at 95°C, the reaction mixture was allowed to cool and stand at room temperature overnight. Addition of acetone caused a white solid to precipitate from solution. After being collected by filtration and washed with acetone, the crude product was redissolved in 2 ml water and precipitated by dropwise addition to 75 ml of vigorously stirring acetone. After two repetitions of this procedure, 582 mg (>100%) of material was obtained; this substance was not significantly contaminated by excess alkylating agent or the corresponding benzyl alcohol. The derivatized cyclodextrin was further purified in 150-mg batches by size-exclusion chromatography on a long Sephadex G-15 column (2.5 × 85 cm). The material was dissolved in the minimum amount of water and eluted with 0.005

M NH_4HCO_3. Baseline separation was not observed, so the fraction containing the desired compound was concentrated by lyophilization and the residue rechromatographed under identical conditions. The combined yield of pure **2d** after two chromatographies was 261 mg (46%). UV (H_2O): λ_{max} 259 nm; ^1H NMR (250 MHz, acidic D_2O) δ: 9.83 (s, 1H, exchanged at neutral pH); 7.78 (d, J = 8 Hz, 2H); 7.13 (d, J = 8 Hz, 2H); 5.78 (d, J = 7 Hz, 2H); 5.07–4.83 (bm, 7H); 3.96–3.33 (bm, not integrated); 3.33–2.65 (m's, not integrated); 2.25 (s, 3H).

3 - (4 - Sulfobenzenemethyl) - 4 - methyl - 5(2 - hydroxyethyl)thiazolium, **3d**. 4-Methyl-5-(2-hydroxyethyl)thiazole (0.239 ml, 2.0 mmol), sodium 4-(bromomethyl)benzenesulfonate (*11*) (0.502 g, 2.0 mmol), and potassium iodide (33.2 mg, 0.2 mmol) were stirred together in 6 ml dry DMF at 95°C under argon for 24 hr. The solvent was removed *in vacuo*, and the residue was taken up in water. The aqueous solution was washed several times with ether and then lyophilized. The crude product (ca. 100%), although very pure by NMR, was recrystallized from methanol/ether to give 0.437 g (56%) of material as off-white crystals; mp 204–206°C; R_f 0.1 [cellulose, 4 : 1 : 5 (v/v/v) BuOH : AcOH : H_2O]; UV (H_2O): λ_{max} 259.18 nm; ^1H NMR (250 MHz, acidic D_2O) δ: 9.61 (s, 1, exchanged rapidly at neutral pH), 7.69 (d, J = 8 Hz, 2H); 7.27 (d, J = 8 Hz, 2H); 5.60 (s, 2H); 3.71 (t, J = 5.7 Hz, 2H); 2.99 (t, J = 5.7 Hz, 2H); 2.25 (s, 3H).

Benzoin condensations catalyzed by thiazolium salts in aqueous DMSO. In a typical experiment, 60 μl phosphate buffer (0.5 M, pH 8.0) was added to 90 μl 25 mM catalyst (in DMSO) under an inert atmosphere. After the addition of 4.5 μl neat benzaldehyde, the vessel was flushed with argon. The reaction mixture was incubated at 30.0°C for 24 hr. The yield of benzoin was determined by analytical reverse-phase HPLC [C_{18} column; 60 : 40 (v/v) MeOH : H_2O].

Kinetics of the benzoin condensation catalyzed by thiazolium salts. The appropriate catalyst (1.2×10^{-5} mol) was placed in an NMR tube and dissolved in 200 μl D_2O and 350 μl DMSO-d_6. After the addition of 48 μl 0.1 N NaOH in D_2O and 24.4 μl neat benzaldehyde, the reaction was flushed with argon and incubated at 50.0°C. At periodic intervals, the 300-MHz ^1H NMR spectrum was recorded. The progress of the reaction was monitored by following the decrease in integrated intensity of the aldehydic proton of the substrate (9.8 ppm) relative to that of an internal standard, or by following the growth of the peak at 6.25 ppm corresponding to the methine proton of the product. Alternately, the course of the reaction could be monitored by analytical reverse-phase HPLC [C_{18} column; 60 : 40 (v/v) MeOH : H_2O]. At periodic intervals the crude reaction mixture was injected directly into the HPLC, and the yield of benzoin was determined from the relative response factors for starting material and product. The latter had been previously determined with authentic mixtures. The second-order rate constants were calculated using a computer program which fit the experimental data according to an integrated second-order rate equation.

Tritiated benzaldehydes. Freshly distilled aldehyde (4.7 mmol) in dry tetrahydrofuran (25 ml) was added to a 0.01 M solution of sodium hydroxide (10 ml). Sodium borotritiide (ca. 2 μmol; ca. 500 μCi) and, after 30 min, unlabeled sodium borohydride (48 mg, 1.25 mmol), were added. After stirring for 2 hr the solution was acidified with 1 N HCl and extracted with ethyl acetate to give the crude [1-

^{3}H]alcohol, which was used without further purification. The latter was dissolved in methylene chloride (1.5 ml) and added to a magnetically stirred solution of pyridinium dichromate (*12*) (2.5 g, 6.8 mmol) in methylene chloride (6 ml). After stirring at room temperature for 10 hr, the reaction mixture was diluted with ether and filtered through a plug of Florisil. The crude, labeled aldehyde obtained upon evaporation of solvent was purified by flash chromatography [silica gel, 2.5 × 14 cm; eluted with 90 : 10 (v/v) hexane : ether]. In this manner [1-^{3}H]benzaldehyde (yield, 56%; sp act, 4.1 × 10^6 cpm/mmol) and [1-^{3}H]*p-tert*-butylbenzaldehyde (yield, 77%; sp act, 5.3 × 10^6 cpm/mmol) were prepared. Both compounds were stored under argon in the refrigerator.

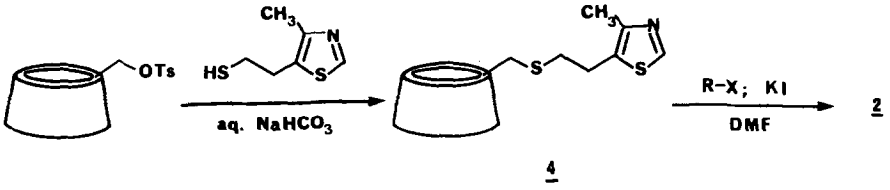
Tritium exchange kinetics. Typically, 400 μl 0.1 M phosphate buffer (pH 8.0) was added under inert atmosphere to the appropriate catalyst (15 μmol) dissolved in 600 μl DMSO. The reaction was initiated by adding 30 μl of a stock solution of tritiated aldehyde (50 mM in DMSO), and thermostated in a constant temperature bath at 50°C under argon. At appropriate intervals 100-μl aliquots were removed by syringe, diluted with 50 μl 1.0 N HCl, and extracted with four 0.5-ml portions of toluene. The combined extract was diluted with 10 ml of PPO/POPOP scintillation solution, and the activity of the sample was determined using a Packard Tri-Carb 460 DC liquid scintillation system. The first-order rate constants were calculated by standard computer techniques from the relative number of cpm per vial. Reproducibility was within 10% between duplicate analysis, while typical correlation coefficients were 0.99 or greater.

Kinetics of the thiazolium-catalyzed oxidation of aldehydes by ferricyanide. Oxidations were performed in 60 : 40 (v/v) DMSO : buffer (aq. 0.5 M phosphate buffer, pH 7.5) at 30.0°C in a 1-ml uv cuvette. In general, 400 μl of a 25 mM stock solution of aromatic aldehyde in DMSO was mixed with 400 μl of a 2.5 mM stock solution of potassium ferricyanide in the aqueous buffer. The reaction was initiated by addition of 200 μl 2.5 mM catalyst in DMSO, and the reduction of ferricyanide was monitored spectroscopically at 420 nm (ε_{420} = 1040 M^{-1} cm^{-1}). The zero-order rate constants for this process were determined by standard linear regression analysis of the experimental data, and corrected by subtraction of the independently measured zero-order rate of catalyst oxidation. The correlation coefficients were 0.99 or greater, and the rates for individual determinations were reproducible within 10–15%, except when the rate of catalyst oxidation accounted for more than 50–60% of the total observed rate. The formation of *p*-nitrobenzoic acid and *p-tert*-butylbenzoic acid from the respective aldehydes was verified by analytical reverse-phase HPLC [C_{18} column, 80 : 20 (v/v) MeOH : H_2O] and comparison with authentic samples. Binding constants were determined kinetically with the cyclodextrin thiazolium catalysts **2b** and **2d** by the Eadie method (*13*).

RESULTS AND DISCUSSION

Synthesis of the Cyclodextrin-Thiazolium Salts

As the initial target for the development of mimics of thiamine-dependent enzymes we chose a β-cyclodextrin derivative **2**, covalently modified at C-6 with a thiazolium salt. The primary side derivatives of cycloamyloses are more accessi-



SCHEME 1

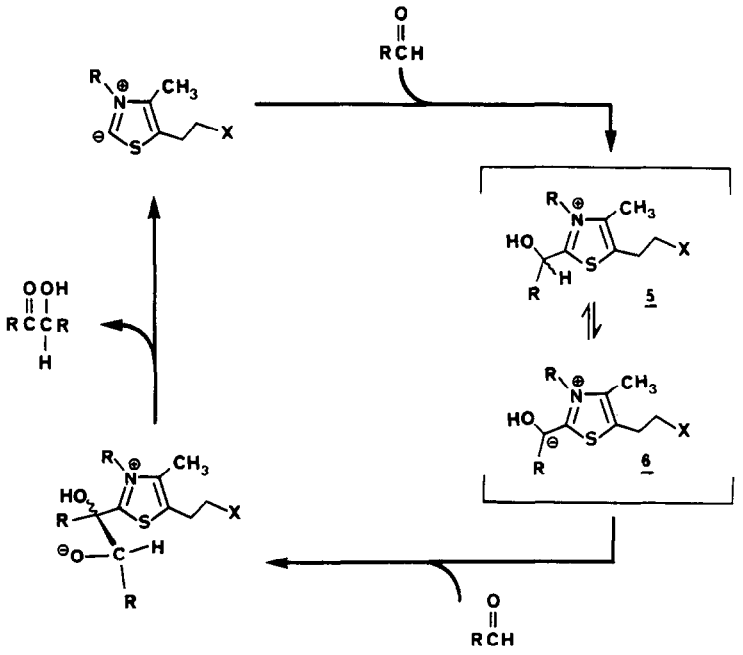
ble synthetically than the secondary side analogs **3**, and model building indicated that the coenzyme, attached to the sugar via the catalytically unimportant 5-hydroxyethyl group, would be able to attack lipophilic substrates bound within the cavity.

As illustrated in Scheme 1, the desired cyclodextrin catalysts were prepared in a straightforward manner from β-cyclodextrin-6-monotosylate in two steps. The latter reacted with 4-methyl-5-(2-mercaptoethyl)thiazole in high yield to give the cyclodextrin thiazole **4**, which was easily recrystallized from water. The TLC behavior and the spectroscopic properties of this material were consistent with the proposed structure; the chromophore absorbs at 252 nm and the ^{1}H NMR shows a singlet at 8.78 ppm which integrates to one proton (relative to the seven anomeric protons of the sugar). In addition, the CI-mass spectrum of the cyclodextrin thiazole showed a cluster of peaks centered around *m/e* 1274 (M-1). Quaternization of the thiazole nitrogen was easily effected by reacting **4** with the appropriate alkyl or benzyl halide and a catalytic amount of potassium iodide in dimethylformamide at elevated temperature. The polar products **2a–c** were purified by ion-exchange chromatography on Sephadex CM-25 eluted with a concentration gradient of NH_4HCO_3 buffer. Ion-exchange chromatography was also subsequently employed to replace the counterion of each of these derivatives with chloride. Since the zwitterionic **2d** was not retained on the Sephadex column, it was purified instead by size-exclusion chromatography on a long G-15 column. The isolated yields of the thiazolium derivatives were typically 40–50%.

The spectral characteristics of **2a–d** were diagnostic for thiazolium salts. Thus, the C-2 proton of each compound appeared in the region 9.5–9.8 ppm in the ^{1}H NMR, and was observed to exchange rapidly in D_2O at neutral pH. The uv spectrum of **2a** was qualitatively the same as that seen for thiamin itself, with two maxima at λ_{max}-269 and 233 nm, the relative intensities of which were pH-dependent. The remaining derivatives **2b–d** had a single peak in the uv region with λ_{max}-259 nm, and an identical 7-nm shift in absorbance maximum relative to the unquaternized thiazole was seen upon reaction of 4-methyl-5-(2-hydroxyethyl)thiazole with the same alkylating agents. Furthermore, the protonated molecule **2a** was readily observed at *m/e* 1397 (M + H − Cl) in the secondary ion mass spectrum (*10*).

Reactions Examined

In order to evaluate the effectiveness of the cyclodextrin derivatives **2**, three catalyzed reactions were investigated. In one of these, the thiazolium-catalyzed benzoin condensation, the expectations were mixed. The generally accepted



SCHEME 2

mechanism of this transformation is shown in Scheme 2 (*4*). Reaction of the first benzaldehyde molecule with the thiazolium salts should be promoted by cyclodextrin binding, but the addition of the *second* benzaldehyde might be more difficult if the intermediate **5** (R = phenyl) was hindered by being bound in the cyclodextrin cavity. In fact, with the exception of **2d**, our cyclodextrin–thiazolium catalysts were inferior to simple thiazolium salts. Two other catalyzed processes require only *one* aldehyde to react; in one study tritium loss from the adduct **5** was examined, while in another study this intermediate was oxidized by ferricyanide. Both of these reactions showed the hoped-for cooperative binding of substrate by cyclodextrin and thiazolium salt.

Thiazolium-catalyzed benzoin condensations. The benzoin condensation is the prototypic reaction effected by thiazolium salts, and a variety of analytical techniques have been applied to its study. For reasons of solubility the condensations catalyzed by **2** and **3** were run in 60 : 40 (v/v) DMSO : aqueous buffer (*14*), and the yields of product were quantified by ^{1}H NMR spectroscopy or analytical reverse-phase HPLC. We found that all the compounds which were prepared promoted the formation of benzoin, albeit to varying extents. After 24 hr at 30°C the yield of product was 32% with **2a** and 10% with **2b**. In contrast, the yields were 52 and 23% with the respective simple thiazolium salts **3a** and **3b**. The fact that higher yields of product were obtained with the *N*-pyrimidylmethyl than with the *N*-ethyl derivatives was consistent with the literature observation that electron-withdrawing groups at N-3 facilitate this thiazolium-catalyzed reaction (4). More significant, however, is the finding that the cyclodextrin derivatives are actually poorer turnover catalysts than their simple analogs lacking the artificial binding site.

As shown in Fig. 1a, similar results were obtained with the N-benzyl salts **2c** and **3c**. A plot of product yield as a function of time for the two reactions revealed that, at longer reaction times, more benzoin was formed with the simple thiazolium salt. However, the amount of benzoin produced in the initial stages of the reaction did not differ significantly for the two catalysts, even though the reaction with the simple thiazolium salt **3c** was performed with a higher concentration of base. The yields for the two reactions qualitatively diverge after formation of approximately one equivalent of benzoin per molecule of catalyst. As shown in

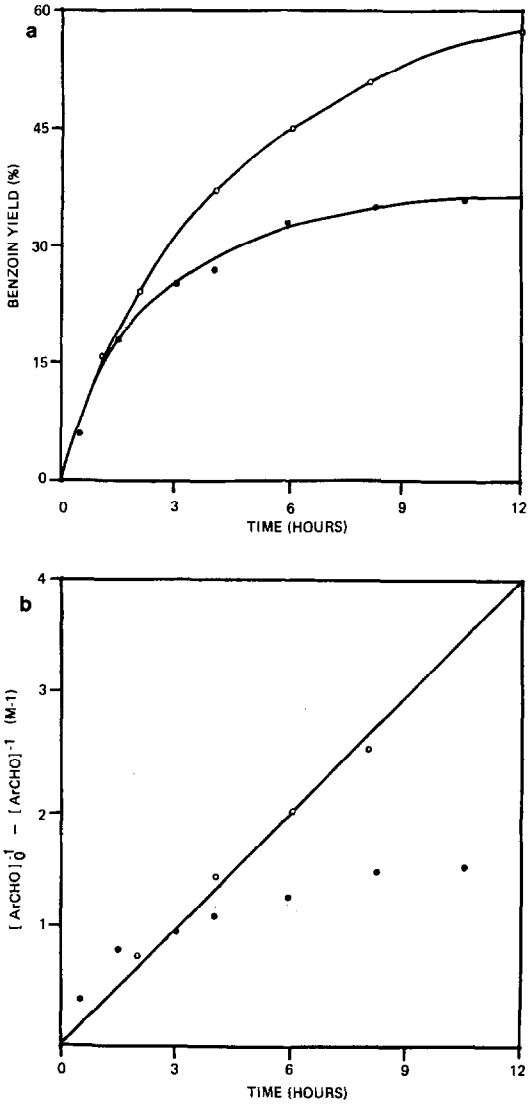


FIG. 1. (a) Plots of benzoin yield vs time. ●, [**2c**] = 20 mM, [ArCHO] = 400 mM, [NaOH] = 8 mM. ○, [**3c**] = 20 mM, [ArCHO] = 400 mM, [NaOH] = 20 mM. In 60 : 40 (v/v) DMSO : H_2O; incubated at 50°C. (b) Second-order plots based on the disappearance of benzaldehyde. Conditions as in (1a).

Fig. 1b, the disappearance of benzaldehyde is cleanly second order in the presence of **3c** ($k_{obs} = 9.4 \times 10^{-5}$ M^{-1} sec^{-1}), while the data obtained with the cyclodextrin thiazolium salt **2c** cannot be fit to a simple integrated second-order rate equation. These observations are not surprising if product inhibition due to competitive binding is important in the reactions promoted by the cycloamylose catalysts. The affinity of benzoin for the apolar cycloamylose cavity is expected to be at least as great as that of the starting material; its presence at the "active site" would inhibit substrate binding and thereby hinder catalyst turnover. Product inhibition has been observed previously in reactions catalyzed by cycloamylose derivatives (*15*), and since additional terms for both substrate and inhibitor binding must be included in the rate equation, simple second-order kinetics are not expected.

An additional factor which could significantly diminish the catalytic effectiveness of the cyclodextrin thiazolium salts is suggested by CPK models. If the N-3 substituent of the compounds **2a–c** were to include into the apolar cycloamylose cavity, substrate binding would again be severely inhibited. To prevent such an interaction, we prepared the cyclodextrin derivative **2d**. We reasoned that the sulfonic acid moiety would increase the hydrophilicity of the N-3 substituent and thus substantially lower the affinity of this group for the hydrophobic cyclodextrin cavity. In fact, **2d** is a very good catalyst of the benzoin condensation; after 24 hr at 30°C a 60% yield of benzoin was obtained, while the analogous simple thiazolium salt **3d** gave only 47%. This result is consistent with our rationalization of the relatively poor performance of the catalysts **2a–c** and, at the same time, underscores the importance of proper catalyst design.

The actual kinetic advantage of the cyclodextrin thiazolium salts might not be manifest in the benzoin condensation for another reason as well. The addition of the α-carbanion of the α-hydroxybenzylthiazolium salt **5** (R = phenyl) to the second molecule of benzaldehyde is believed to be partially rate determining (*16*). Since the cycloamylose derivatives do not possess discrete binding sites for both molecules of benzaldehyde, the formation of this new carbon–carbon bond would not be expected to be accelerated significantly by any of our catalysts, and might even be retarded because of steric constraints on the cyclodextrin-bound intermediate. For this reason we decided to determine the rates of thiazolium-catalyzed isotope exchange of tritiated aromatic aldehydes which requires reaction of a single substrate molecule with catalyst.

Thiazolium-catalyzed isotope exchange of [^{3}H]benzaldehydes with solvent. Thiazolium salts promote the exchange of aldehydic protons (*17*), so that the rate of formation of the "active aldehyde" intermediate **5** can be measured by monitoring the time-dependent loss of tritium from suitably labeled substrates. Aromatic aldehydes were employed in these studies, since the kinetic acidity of the α proton of the α-hydroxybenzyl thiazolium adducts is great enough to allow for convenient assay of the reaction over reasonable periods of time (*17*). Moreover, these molecules were predicted to be especially good substrates for the cyclodextrin catalysts because of their affinity for the apolar cycloamylose cavity. Our earlier investigations of the cyclodextrin–pyridoxamine systems had suggested that cooperative effects would not be observed with simple aliphatic aldehydes,

TABLE 1

THIAZOLIUM-CATALYZED TRITIUM EXCHANGE OF [1-^{3}H]BENZALDEHYDE

	2 k (hr^{-1})	**3** k' (hr^{-1})	k/k'
a	—	—	—
b	—	—	—
c	0.292 ± 0.017	0.103 ± 0.003	2.8
d	0.246 ± 0.004	0.106 ± 0.003	2.3

Note. [cat] = 15 mM in 60:40 (v/v) DMSO:aqueous phosphate buffer (0.1 M, pH 8.0); incubated at 50°C.

like acetaldehyde, which bind poorly (*2*, *3*). In order to test whether the cyclodextrin catalysts might exhibit more finely tuned substrate selectivity, two tritiated compounds were prepared, [1-^{3}H]benzaldehyde and [1-^{3}H]4-*tert*-butylbenzaldehyde. We anticipated that the latter compound would be a better substrate for the holoenzyme mimics, since aromatic rings containing *tert*-butyl substituents are known to bind more strongly to β-cyclodextrin than simple unsubstituted benzenes (*18*).

The exchange studies were carried out in aqueous DMSO under conditions of catalyst in excess. The evaluation of each cyclodextrin derivative **2a–d** and that of its simple analog **3a–d** were carried out in parallel. The loss of tritium from the substrate aldehyde was measured by scintillation counting, and good first-order kinetics were obtained for each of the catalysts over the time periods examined. Our results are summarized in Tables 1 and 2.

Although the relative rate enhancements under these conditions are generally modest, the cyclodextrin thiazolium salts were the superior catalysts in all the

TABLE 2

THIAZOLIUM-CATALYZED EXCHANGE OF [1-^{3}H]4-*tert*-BUTYLBENZALDEHYDE

	2 k (hr^{-1})	**3** k' (hr^{-1})	k/k'
a	0.371 ± 0.005	0.105 ± 0.003	3.5
b	0.395 ± 0.018	0.092 ± 0.004	4.3
c	0.155 ± 0.007	0.088 ± 0.004	1.8
d	0.760 ± 0.020	0.076 ± 0.001	10.0

Note. [cat] = 15 mM; [4-*tert*-butylbenzaldehyde] = 1.5 mM in 60:40 (v/v) DMSO:aqueous phosphate buffer (0.1 M, pH 8.0); incubated at 50°C.

cases examined. It is significant that the observed accelerations were largest with the substrate *p-tert*-butylbenzaldehyde, which supports the notion that higher local concentrations of this material are attainable because of its greater affinity for the binding site. Furthermore, the largest rate enhancements were found for the catalyst **2d**, which contains the sulfonic acid moiety to prevent competitive binding of the N-3 substituent. This thiazolium derivative was also the best catalyst of the benzoin condensation.

The rate accelerations obtained under this given set of experimental conditions do not necessarily reflect the maximum kinetic advantage of the cyclodextrin thiazolium salts. A better estimate of the catalytic efficiency of our holoenzyme mimics requires complete evaluation of the kinetic parameters for the exchange process, but generation of the required amount of information using the tritium exchange protocol would represent a considerable undertaking. As described in the following section, the reported (*19*) rapid oxidation of the α-hydroxy carbanion provides a basis for more convenient spectroscopic investigation of this problem.

Thiazolium-catalyzed oxidation of aldehydes by ferricyanide. In the presence of substrate and a suitable oxidizing agent like ferricyanide, the enzyme yeast pyruvate decarboxylase forms acetate (*20*). This process involves the oxidation of the α-hydroxyethyl carbanion intermediate **6** (Scheme 2, R = methyl), followed by rapid solvolysis of the acyl thiazolium. Although initial reports indicated that the oxidation of **5** was negligible in the absence of enzymes (*21*), subsequent studies have shown that thiazolium salts can promote the oxidation of aldehydes in the presence of a variety of oxidizing agents in free solution. Aromatic nitro compounds (*22*), quinones (*23*), various redox dyes (*24*), as well as a flavin–micelle system (*19*) have been successfully used as oxidants. We now find that the oxidation of aromatic aldehydes by ferricyanide is also catalyzed by thiazolium salts in 60 : 40 (v/v) DMSO : aqueous phosphate buffer (buffer, pH 7.5). The course of the reaction can be conveniently followed by monitoring the decrease in absorbance of the Fe(III) chromophore at 420 nm. Thus, *p*-nitrobenzaldehyde is rapidly oxidized by ferricyanide to *p*-nitrobenzoic acid in the presence of the simple *N*-benzyl thiazolium salt **3c** at 30.0°C. The identity of the product acid was confirmed by comparison with an authentic sample using analytical HPLC. This reaction was determined to be zero-order in oxidant and first-order in both substrate and catalyst. In the absence of the thiazolium salt, ferricyanide is not reduced. Unfortunately, however, the catalyst itself is also oxidized under the experimental conditions, so that the rates determined in the presence of an aldehyde substrate must be corrected by subtraction of this independently measured background rate. The competing oxidation of catalyst presumably involves the well-known ring-opening reaction of thiazolium salts to give an easily oxidized thiolate species (*25*). Under typical reaction conditions the rate of oxidation of **3c** is ca. 20 times slower than the total rate observed in the presence of *p*-nitrobenzaldehyde. The *N*-benzyl thiazolium cyclodextrin compound **2c** also catalyzes the oxidation of this substrate. After making the appropriate corrections for the background rate, a ca. twofold enhancement in rate is found for the reaction promoted by this cyclodextrin catalyst relative to **3c**. A similar trend is observed for the catalyst pair **2d**/

3d; the reaction facilitated by the cyclodextrin derivative is accelerated by a factor of 2.3.

This spectroscopic assay for the formation of the "active aldehyde" intermediate provides a useful tool for the study of saturation phenomena with the cyclodextrin catalysts. Since the largest rate enhancements were observed with 4-*tert*-butylbenzaldehyde in the tritium exchange work, this substrate was employed for these investigations as well. The oxidation of *tert*-butylbenzaldehyde is significantly slower than that of 4-nitrobenzaldehyde under identical experimental conditions, so that the correction for the competing oxidation of catalyst accounts for a much larger portion of the total observed rate of reduction of ferricyanide. The determination of the aldehyde oxidation rate is, consequently, subject to considerable error, especially when the correction is of the same magnitude as the total measured rate. When the reaction promoted by the cyclodextrin derivatives was run with substrate in excess, however, the oxidation rates could be reproduced with an accuracy of 10–15%. Saturation kinetics were observed with both **2c** and **2d** upon variation of the substrate concentration, and standard Eadie treatment (*13*) of the data yielded linear plots (correlation coefficients greater than 0.97), from which the kinetic parameters k_{cat} and K_M could be derived. For the *N*-benzyl catalyst **2c** the values $k_{cat} = 0.040 \pm 0.003$ min^{-1} and $K_M = 5.2 \pm 0.6$ mM were calculated; for the *p*-sulfobenzyl thiazolium cyclodextrin **2d** the values were $k_{cat} = 0.067 \pm 0.004$ min^{-1} and $K_M = 4.8 \pm 0.4$ mM. The dissociation constants measured for the complexes of 4-*tert*-butylbenzaldehyde with these cyclodextrin derivatives are the same within experimental error and, in addition, are comparable to that reported (*14*) for the complex of *m-tert*-butylphenylacetate with β-cyclodextrin itself in 50% (v/v) DMSO : H_2O ($K_M \simeq 2$ mM). Thus, the kinetic advantage of the *p*-sulfobenzyl compound is reflected entirely in the k_{cat} term.

The kinetic parameters which have been calculated for the two catalysts **2c** and **2d** were used in the Michaelis–Menten equation, together with the correct concentrations of substrate and catalyst, to predict the initial rates of tritium exchange. Thus, a rate of 4.46×10^{-5} M min^{-1} was predicted for the reaction catalyzed by **2c**, which compares with the actual observed rate of 9.9×10^{-6} M min^{-1} (see Table 2 in the previous section). The difference in these values corresponds to a tritium isotope effect of $k_H/k_T = 4.5 \pm 0.3$, which agrees well with the value of 4.3 obtained by Schowen for the analogous step in the cyanide-catalyzed benzoin condensation (*26*). Similarly, an isotope effect of 4.0 ± 0.3 was found for the catalyst **2d**; an initial rate of 7.57×10^{-5} M min^{-1} was predicted, and a value of 1.88×10^{-5} M min^{-1} was observed.

The maximum kinetic advantage of the cyclodextrin binding site for a particular substrate can be estimated by comparing the apparent bimolecular rate constant k_{cat}/K_M calculated for the enzyme mimics **2c** and **2d** with the second-order rate constant of the reaction between that substrate and the corresponding simple thiazolium salts **3c** and **3d**. Using the spectroscopic method described, we were unable to measure the bimolecular rate constants accurately for the reaction of 4-*tert*-butylbenzaldehyde with the simpler *N*-alkyl thiazolium salts **3c** and **3d**. The rate of catalyst oxidation typically accounted for 60–90% of the total rate of reduction of ferricyanide, so the errors in correction become considerable. Never-

theless, the second-order rate constants could be estimated from the tritium exchange data to be 0.102 and 0.0803 M^{-1} min^{-1} for **3c** and **3d**, respectively, since this reaction is first order in catalyst. Assuming the same kinetic isotope effect seen with the respective cyclodextrin analogs, the values of k_{obs} would be 0.460 and 0.332 M^{-1} min^{-1}, respectively. Comparing these estimated second-order rate constants with the respective k_{cat}/K_M values, the rate acceleration caused by linkage to the cyclodextrin is 17-fold for the *N*-benzyl thiazolium cyclodextrin, while that of the *N*-*p*-sulfobenzyl thiazolium cyclodextrin is 43-fold.

CONCLUSIONS

The results described in the foregoing sections demonstrate that significant cooperative catalysis can be achieved between a cyclodextrin binding site and a covalently attached thiazolium moiety. Unlike the previously reported cyclodextrin transaminase mimics which require continual chemical regeneration, the readily accessible thiazolium derivatives are true turnover catalysts. When properly designed, they effectively promote reactions which require the biological cofactor thiamine pyrophosphate. The processes we have examined exhibit several features typical of real enzymes, including substrate selectivity, saturation kinetics, and catalytic rate enhancements. We anticipate that incorporation of other catalytic groups to facilitate the required proton removals and optimization of transition state geometry by finding better-fitting substrates will lead to even more successful mimics of thiamine-dependent enzymes.

REFERENCES

1. Breslow, R. (1982) *Science* **218,** 532.
2. Breslow, R., Hammond, M., and Lauer, M. (1980) *J. Amer. Chem. Soc.* **102,** 421.
3. Breslow, R., and Czarnik, A. W. (1980) *J. Amer. Chem. Soc.* **105,** 1390.
4. Breslow, R. (1958) *J. Amer. Chem. Soc.* **80,** 3719.
5. Stetter, H., and Kuhlmann, H. (1976) *Chem. Ber.* **109,** 2890.
6. Stetter, H., and Kuhlmann, H. (1975) *Synthesis* 379.
7. Gibbs, E. M., and Robinson, F. A. (1945) *J. Chem. Soc.* 925.
8. Chao, Y. (1972) Ph.D. Thesis, Columbia University.
9. Andersag, H., and Westphal, K. (1937) *Chem. Ber.* **70,** 2035.
10. Kambara, H., Ogawa, Y., Mochizuki, K., and Hishida, S. (1982) *Mass Spectrosc.* **30,** 169.
11. Hubbuch, A., Bindewald, R., Fohles, J., Naithani, V. K., and Zahn, H. (1980) *Angew. Chem.* **92,** 394.
12. Corey, E. J., and Schmidt, G. (1979) *Tetrahedron Lett.* 399.
13. Eadie, G. S. (1942) *J. Biol. Chem.* **146,** 85.
14. Siegel, B., and Breslow, R. (1975) *J. Amer. Chem. Soc.* **97,** 6869.
15. Lipsey, C. (1980) Ph.D. Thesis, Columbia University.
16. Yano, Y., Tamura, Y., and Tagaki, W. (1980) *Bull. Chem. Soc. Japan* **53,** 740.
17. Gallo, A. A., and Sable, H. Z. (1976) *J. Biol. Chem.* **251,** 2564.
18. Breslow, R., Czarniecki, M. F., Emert, J., and Hamaguchi, H. (1980) *J. Amer. Chem. Soc.* **102,** 762.

19. SHINKAI, S., YAMASHITA, T., KUSANO, Y., AND MANABE, O. (1982) *J. Amer. Chem. Soc.* **104,** 563.
20. HEALY, M. J., AND CHRISTEN, P. (1973) *Biochemistry* **12,** 35.
21. KRAMPITZ, L. O., SUZUKI, I., AND GRUELL, G. (1961) *Fed. Proc.* **20,** 971.
22. CASTELLS, J., PUJOL, F., LLITJOS, H., AND MORENO-MAÑAS, M. (1982) *Tetrahedron* **38,** 337.
23. VOVK, A. I., BABICHEVA, A. F., AND YASNIKOV, A. A. (1976) *Dopov. Akad. Nauk. Ukr. RSR, Ser. B.* 1094; (1977) *Chem. Abst.* **86,** 67430j.
24. GOLDSTEIN, T. P. U.S. Patent No. 3816522, June 11, 1974.
25. EL HAGE CHAHINE, J.-M., AND DUBOIS, J.-E. (1983) *J. Amer. Chem. Soc.* **105,** 2335.
26. KUEBRICH, J. P., SCHOWEN, R. L., WANG, M.-S., AND LUPES, M. E. (1971) *J. Amer. Chem. Soc.* **93,** 1214.