Amperometric Assay for Aldolase Activity: Antibody-Catalyzed Ferrocenylamine Formation

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Screening of new catalysts for aldolase activity is a major task in bioorganic chemistry. For this purpose, fast and convenient methods are required for the detection of the catalysts. We have developed the first amperometric assay for aldol or retro-aldol catalytic activity. A new ferrocene-aldol derivative was synthesized with redox activity significantly different from that of ferrocenylamine. It was shown that the reaction between aldolase antibody 38C2 and a ferrocene-aldol substrate generated free ferrocenylamine, which could be detected and quantified by simple electrochemical measurement. The amperometric assay was applied to perform a Michaelis−Menten analysis of catalytic antibody 38C2 in order to determine the enzymatic kinetic parameters.

The aldol reaction is one of the most important C−C bond-forming reactions employed in synthetic transformations. Therefore, the detection of new organic and bioorganic aldol catalysts remains an important task. Aldolase antibody 38C2 is perhaps the most promiscuous antibody catalyst generated to date.[1] The antibody was found to efficiently catalyze a remarkable substrate scope of aldol and retro-aldol reactions through an enamine class I aldolase mechanism.[2,3] The tandem retro-aldol retro-Michael reaction catalyzed by antibody 38C2 was demonstrated to be an efficient cleavage reaction for the concept of prodrug activation.[4−10]

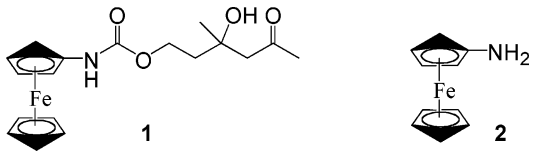
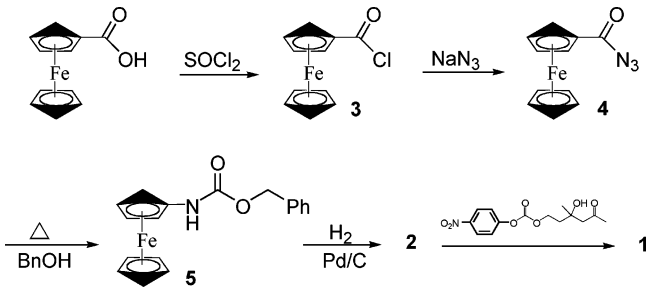


Figure 1. Chemical structures of retro-aldol retro-Michael substrate **1** vs reaction product **2**.

Scheme 1. Chemical Synthesis of Ferrocenylamine 2 and Substrate 1



This reaction is not known to be catalyzed by any natural enzymes, and therefore, nonspecific prodrug activation by endogenoese enzymes could be avoided.[11] Since the aldol addition is a reversal reaction, aldol catalysts can usually also catalyze the retro-aldol reaction. Therefore, screening for retro-aldol catalysts could be a convenient route for finding new aldol catalysts. Several variations of fluorogenic assays were developed for aldolase activity,[12−14] including one direct visual detection.[15] Here we report the first amperometric assay, based on the formation of the electrochemically active ferrocenylamine, for the detection of aldolase activity.

An amperometric assay is usually based on electric current formation at a certain potential upon the oxidation or reduction of an electrochemically active compound. The assay conditions require such a substrate, which has an oxidation−reduction potential different from that of its product that is formed by the catalyst. In other words, current can be detected only upon the formation of the reaction product. Substrate **1**, (Figure 1) designed

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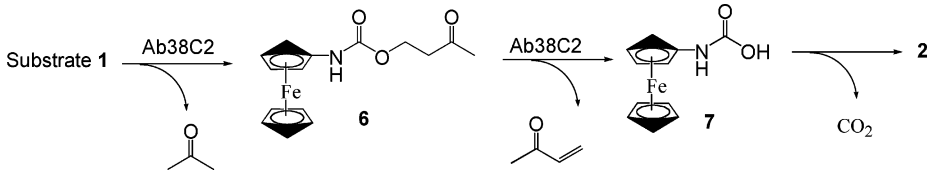
Scheme 2. Release of Ferrocenylamine 2 by Antibody 38C2 from Substrate 1



to detect retro-aldol retro-Michael activity, is a carbamate derivative of ferrocenylamine **2**. Therefore, the iron(II) center in **2** has a higher electron density around it and, thus, is expected to be oxidized at a lower potential than carbamate **1**.

RESULTS AND DISCUSSION

Ferreocenylamine **2** was synthesized according to a known procedure with a slight modification[16] (Scheme 1). Thus, ferrocenecarboxylic acid was reacted with thionyl chloride to form acyl chloride **3**, which was further reacted with sodium azide to give acyl azide **4**. The latter was refluxed in toluene in the presence of benzyl alcohol to form benzylcarbamate **5**, which was then deprotected by hydrogenation to generate ferreocenylamine **2**. Substrate **1** was obtained by direct reaction of amine **2** with the 4-nitrophenyl carbonate derivative of the retro-aldol retro-Michael linker.

Substrate **1** was initially incubated with antibody 38C2 in phosphate-buffered saline (PBS) pH 7.4. It was efficiently cleaved by antibody 38C2 according to Scheme 2, and ferrocenylamine release was detected by RP-HPLC. No release was observed when the reaction was performed in the buffer without the antibody.

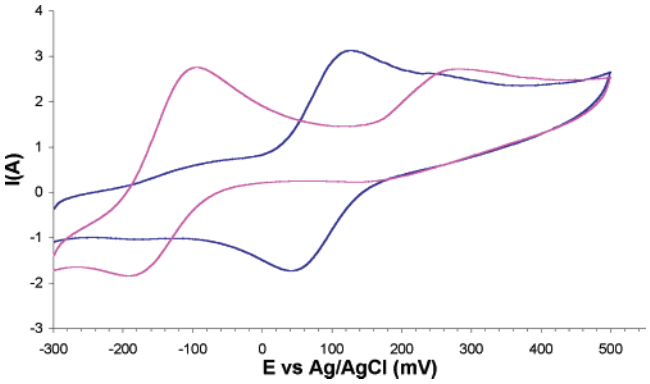


Figure 2. Cyclic voltammograms obtained for (red) ferrocenylamine **2** [160 μM] and (blue) substrate **1** [160 μM].

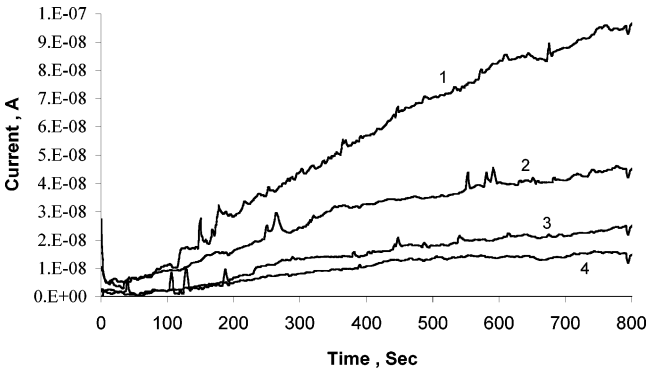


Figure 3. Amperometric response to ferrocenylamine released from substrate **1** (1.0 mM) after the addition of 38C2 in different concentrations: (1) 6.6, (2) 3.3, (3) 0.66, and (4) 0.33 μM.

Next, we measured the cyclic voltammograms of substrate **1** and ferrocenylamine **2** (Figure 2). Expectedly, ferrocenylamine exhibited redox behavior at lower potentials than its carbamate derivative. The anodic oxidation peak of **2** is ~150 mV lower than that of substrate **1**. Antibody 38C2 was then added in four different concentrations to a 1 mM solution of **1** in PBS, pH 7.4. The current was monitored at a constant potential of −30 mV, and the measurements are shown in Figure 3. The plots indicate the formation rate of ferrocenylamine **2** from substrate **1** as a result of the antibody catalytic activity. Current change could still be detected for a reaction at antibody concentration as low as 0.33 μM.

In view of the latter results, we decided to perform a Michaelis−Menten analysis, based on the amperometric response to ferrocenylamine release, and check whether one can determine the kinetic parameters K_m and k_{cat}. Five solutions of substrate **1** in various concentrations were incubated with catalytic antibody 38C2, and the initial rate release was measured with an amperometric cell (see Experimental Section). To our delight, the Lineweaver−Burk plot was obtained with an excellent correlation (R^2 value of 0.9985) and the kinetic constants K_m and k_{cat} were calculated to be 375 ± 19 μM and 0.24 ± 0.012 min^{-1}, respectively (Figure 4). These numbers agree with other Michaelis−Menten constants measured for similar substrates of antibody 38C2[3].

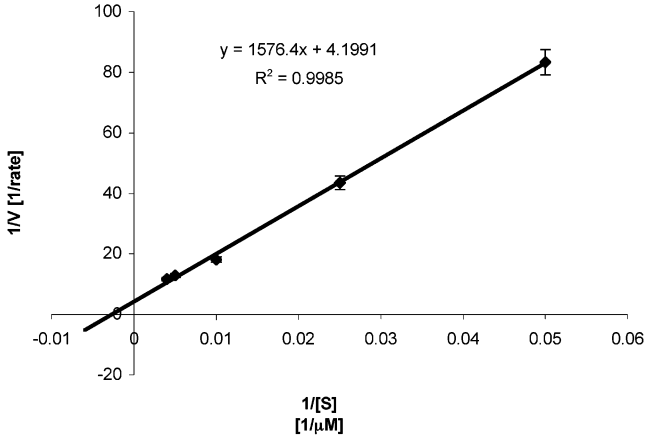


Figure 4. Lineweaver−Burk plot for antibody 38C2-catalyzed ferrocenylamine release from substrate **1**. For reaction conditions, see Experimental Section.

The most common amperometric substrate, based on a release of a redox-active molecule, is a 4-aminophenol derivative.[17] For example, esterification of its phenol group with phosphoric acid generates a substrate for an amperometric assay of alkaline phosphatase activity. The measured oxidation potential of 4-ami-

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nophenol in our assay conditions was 70 mV, which is 170 mV higher than the oxidation potential of ferrocenamine **2** (−100 mV). This potential difference indicates that an assay based on a ferrocenamine release may have an advantage over 4-aminophenol release. In general, measurements at lower potentials are preferred in order to prevent undesired oxidation reactions of other species that could be present in the reaction mixture. As far as we know, ferrocene compounds were never used in an electrochemical assay in which a molecule, with redox activity different from its parent substrate, is released. The concept of ferrocenylamine release could also be applied for screening of other catalytic activities. For example, preparation of a specific amide derivative of ferrocenylamine will generate a substrate for an amperometric assay of a specific protease activity.

In summary, we have described the first amperometric assay for aldolase activity. A new ferrocene aldol derivative was synthesized with a redox activity significantly different from that of ferrocenylamine. It was shown that the reaction between aldolase antibody 38C2 and ferrocene aldol **1** generated free ferrocenylamine, which could be detected and quantified by a simple electrochemical measurement. The amperometric assay was applied to perform a Michaelis−Menten analysis in order to determine the enzymatic kinetic parameters. Specifically, the studied retro-aldol retro-Michael reaction assay could be applied to discover new improved biocatalysts with aldolase activity.

EXPERIMENTAL SECTION

General Methods. Thin-layer chromatography was performed using Merck 60 F_{254} silica gel plates, compounds were visualized by irradiation with UV light or by treatment with a solution of 25 g of phosphomolybdic acid, 10 g of $Ce(SO_4)_2 \cdot H_2O$, 60 mL of concentrated H_2SO_4, and 940 mL of H_2O followed by heating, by staining, or both, with a solution of 12 g of 2,4-dinitrophenylhydrazine in 60 mL of concentrated H_2SO_4, 80 mL of H_2O, and 200 mL of 95% EtOH followed by heating. Flash chromatography was performed using silica gel Merck 60 (particle size 0.040−0.063 mm), eluent given in parentheses. ^{1}H NMR spectra were measured using Bruker Avance operated at 200 or 400 MHz as mentioned. ^{13}C NMR spectra were measured using Bruker Avance operated at 50 or 100 MHz as mentioned. The chemical shifts are expressed in δ relative to TMS ($\delta = 0$ ppm) and the coupling constants J are in hertz. The spectra were recorded using $CDCl_3$ as a solvent at room temperature unless stated otherwise. Catalytic antibody 38C2 was obtained from The Scripps Research Institute, La-Jolla, Ca. All general reagents, including salts and solvents, were purchased from Aldrich (Milwaukee, MN). All reactions were carried out at room temperature unless stated otherwise.

Synthesis. Ferrocenylamine 2. Acylazidoferrocene[16] **4** (150 mg, 0.59 mmol) was dissolved in toluene (2 mL). The solution was heated to 105 °C, and benzyl alcohol (0.12 mL, 1.18 mmol) was added in a single portion. The solution was refluxed for an additional 2 h. The mixture was cooled to room temperature, the solvent was evaporated under reduced pressure, and the crude mixture was purified by flash chromatography (5% ethyl acetate (EtOAc)/95% *n*-hexane (Hex)) to give the carbamate derivative **5** as a reddish solid (170 mg, 86%). Compound **5** (163 mg, 0.48 mmol) was dissolved in MeOH (5 mL), and a catalytic amount of palladium on activated carbon was added. The mixture was stirred under hydrogen for 1 h, the crude mixture was filtered, the solvent was evaporated under reduced pressure, and the resulting material was purified by flash chromatography (20% EtOAc/80% Hex) to give the desired product in the form of a brown solid (96 mg, 99%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.05 (5H, s), 3.95 (2H, s), 3.80 (2H, s), 2.53 (2H, br); ^{13}C NMR (100 MHz, $CDCl_3$) δ 58.59, 63.26, 68.67, 105.30; EI+ calcd for $C_{10}H_{11}NFe$ m/z 201.0.

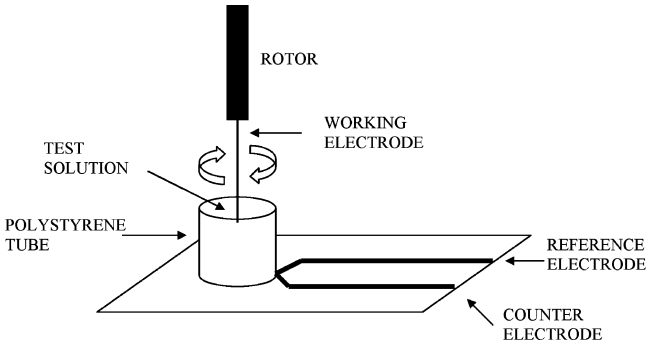

Figure 5. Schematic layout of the electrochemical cell.

Aldol 1. 4-Nitrophenyl carbonate derivative of the retro-aldol retro-Michael linker[9] (100 mg, 321 μmol) was dissolved in dimethylformamide (2 mL). Ferrocenylamine was added (71 mg, 353 μmol), followed by 1-hydroxybenzotriazole hydrate (16 mg, 118 μmol) and diisopropylethylamine (180 μL). The reaction mixture was stirred overnight at 50 °C.The resulting brown mixture was washed with brine, dried over magnesium sulfate, and purified by flash chromatography (50% EtOAc/50% Hex) to give aldol **1** (35 mg, 30%) as an orange solid.

^{1}H NMR (200 MHz, $CDCl_3$) δ 1.25 (3H, s), 1.90 (2H, t, $J = 7.0$ Hz), 2.17 (3H, s), 2.65 (2H, d), 3.97 (2H, s), 4.15 (5H, s), 4.26 (2H, t, $J = 6.85$), 4.44 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 31.6, 33.8, 42.3, 54.43, 62.72, 63.4, 66.3, 71.1, 71.14, 72.5, 212.6; EI+ calcd for $C_{12}H_{23}O_4NFe$ m/z 373.2.

Electrochemical Measurements. Figure 5 shows the schematic layout of the electrochemical cell. All measurements were performed in the three-electrode cell ($V_{solution} = 0.3$ mL) with a rotating cylindrical graphite working electrode (0.9-mm diameter), a graphite ink counter, and Ag/AgCl ink reference electrodes, using PBS (50 mM, pH 7.4) as a solvent. We used a BAS cv-50w potentiostat interfaced to a personal computer system with BAS 50 software. The rotation of the graphite electrode was performed with a Pine instrument rotator and an MSRS speed controller. The kinetic measurements were performed at a fixed electrode potential (−30.0 mV versus Ag/AgCl). Cyclic voltammetry was conducted at a 20 mV/s scan rate.

To produce a Lineweaver−Burk plot, solutions with different concentrations of aldol **1** were prepared (20−250 μM) and the current was measured 200 s after the addition of catalytic antibody 38C2 to a final concentration of 1 mg/mL.

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