Cofactor-Induced Refinement of Catalytic Antibody Activity: A Metal-Specific Allosteric Effect

M. G. Finn, Richard A. Lerner,* and Carlos F. Barbas, III*

Department of Chemistry, University of Virginia Charlottesville, Virginia 22901
The Skaggs Institute for Chemical Biology and Department of Molecular Biology, The Scripps Research Institute, 10650 North Torrey Pines Rd. La Jolla, California 92037

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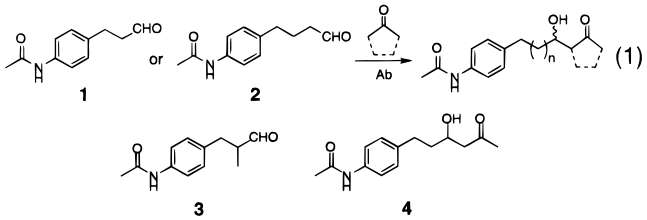
We recently described the development of the strategy of reactive immunization for the production of two catalytic antibodies for the aldol reaction.[1] These catalysts accept a tremendous variety of substrates in aldol addition, aldol condensation, and retro-aldol reactions.[2] They also catalyze a mechanistically related decarboxylation of β-keto acids.[3] A striking example of their effective promiscuity is the highly enantioselective intramolecular aldol condensation to give the Wieland−Miescher ketone, using a catalyst developed by immunization with a hapten only remotely similar to the reaction substrate.[4]

An X-ray crystal structure of one of these aldol catalysts (antibody 33F12) has revealed the presence of a single lysine side chain at the bottom of a large binding pocket lined with hydrophobic residues.[2a] This is consistent with evidence that catalysis occurs via enamine intermediates,[1,2] characteristic of class I aldolases.[5] Along with the ability of metal complexes to assist in the biocatalysis of the aldol reaction, the presence of potential metal-binding groups from several tyrosine and tryptophan residues in the putative antibody active site prompted us to screen the reactivities of these systems with a variety of added metal ions. We report here that "hard" metal ions typically utilized by class II aldolases do not perturb catalysis of the aldol reaction by our antibodies. In contrast, palladium(II) additives improve the rate and enantioselectivity of the process, but only for substrates closely resembling the hapten used for the generation of the antibody.

The reaction of aldehyde **1** with acetone (eq 1)[1] was used to screen the effect of added metal ion. Initial rates in the presence of the following species at 20, 50, and 150 μM were found to be indistinguishable (±5%) from the rates of the aldol reaction in the absence of additive under a standard set of conditions in the saturation regime of catalytic turnover (2 μM antibody;[6] 100 μM **1**; 5% (v/v) acetone; phosphate/saline (PBS) buffer at pH 7.4; 22 ± 1 °C): LiCl, $MgNO_3$, $CaSO_4$, $BaCO_3$, $ZrOCl_2$, $CrCl_2$, $CrCl_3$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $RuCl_3$, $CoCl_2$, $NiCl_2$, K_2PtCl_4, $PtCl_4$, $CuCl_2$, $ZnCl_2$, $CdCl_2$, $HgCl_2$ $CeCl_3$, $LaCl_3$, $La(OTf)_3$, $Eu(OTf)_3$, $YbCl_3$, and $Yb(OTf)_3$. Visible precipitation of the antibodies, with concomitant reduction of the apparent reaction rate, often occurs at metal ion concentrations higher than 150 μM. In contrast,



(1)

$VOSO_4$ at relatively high concentration (100−300 μM relative to 2−5 μM antibody) was found to inhibit the aldol reactions of **1** and **2** with acetone in direct proportion to the amount of metal added,[7] without antibody precipitation.[8]

Palladium(II) is the only metal species tested to have a significant effect on the antibody system at low concentrations. Thus, the standard aldol reaction (**1** + acetone), catalyzed by both 38C2 and 33F12 (2 mM), is *accelerated* by a variety of Pd^{II} salts [K_2PdCl_4 and Na_2PdCl_4, Figure 1A; $Pd(OAc_2)$ and $PdSO_4$, data not shown] and palladium amine complexes [$Pd(en)Cl_2$ and *trans*-$Pd(NH_3)_2Cl_2$, Figure 1B,C]. The magnitude of the relative rate (k_{rel})[9] is roughly proportional to metal concentration below 20 mM, often remaining detectable with as little as a 2-fold excess of Pd with respect to the concentration of antibody active sites.[10] Higher concentrations of Pd^{II} lead to apparent reaction inhibition or attenuation of the acceleratory effect, which is attributed to removal of active antibody from the reaction mixture by increased precipitation as metal concentrations are raised.[11] $Pd(en)Cl_2$ (en = ethylenediamine) consistently induces much less observable precipitation than the other palladium species; accordingly, accelerated rates are observed at substantially higher concentrations of this complex than for other Pd sources. Pd-induced rate accelerations are also observed at higher antibody concentrations and in water instead of PBS buffer.[7]

Michaelis−Menten kinetic parameters measured for the 38C2-catalyzed reaction reveal an improvement in k_{cat} and a small increase in K_m in the presence of Pd^{II} (Table 1). Control experiments show that $Pd(en)Cl_2$−antibody binding is reversible[12] (dissociation constant ≈ 1 μM)[13] without causing functional

(1) Wagner, J.; Lerner, R. A.; Barbas, C. F., III *Science* **1995**, *270*, 1797.
(2) (a) Barbas, C. F., III; Heine, A.; Zhong, G.; Hoffmann, T.; Gramatikova, S.; Björnestedt, R.; List, B.; Anderson, J.; Stura, E. A.; Wilson, E. A.; Lerner, R. A. *Science* **1997**, *278*, 2085. (b) Hoffmann, T.; Zhong, G.; List, B.; Shabat, D.; Anderson, J.; Gramatikova, S.; Lerner, R. A.; Barbas, C. F., III. *J. Am. Chem. Soc. J. Am. Chem. Soc.* **1998**, *120*, 2768. (c) List, B.; Shabat, D.; Barbas, C. F., III; Lerner, R. H. *Chem. Eur. J.* In press.
(3) Björnestedt, R.; Zhong, G.; Lerner, R. A.; Barbas, C. F., III *J. Am. Chem. Soc.* **1996**, *118*, 11720.
(4) Zhong, G.; Hoffmann, T.; Lerner, R. A.; Danishefsky, S.; Barbas, C. F., III. *J. Am. Chem. Soc.* **1997**, *119*, 8131.
(5) Rutter, W. J. *Fed. Proc. Am. Soc. Exp. Biol.* **1964**, *23*, 1248. Hill, H. A. O.; Lobb, R. R.; Sharp, S. L.; Stokes, A. M.; Harris, J. I.; Jack, R. S. *Biochem. J.* **1976**, *153*, 551. Morris, A. J.; Tolan, D. R. *Biochemistry* **1994**, *33*, 12291 and references therein.
(6) All antibody concentrations are reported as the concentration of active sites; an intact IgG antibody molecule contains two active sites.
(7) See the Supporting Information for details.
(8) Removal of small amounts of antibody from the reaction by precipitation, which could account for the diminished rates, was ruled out by the observation that antibody samples treated with $VOSO_4$, filtered, and then subsequently dialyzed to remove the metal operated with undiminished catalytic efficiency. Inhibition by vanadyl ion is on the order of 10−50% for $VOSO_4$ concentrations of 100−400 μM, in the presence of catalytic antibodies at 2.0 and 5.0 μM.
(9) Relative rate is defined as the ratio of the initial reaction rates measured in the presence and absence of the additive under otherwise identical conditions.
(10) No independent metal-mediated aldol reaction occurs in the absence of antibody, even at concentrations of Pd^{II} compounds (500 μM) much higher than those used in the presence of antibody. The pH of the reaction mixtures in PBS buffer are unchanged by the addition of Pd^{II} salts at the concentrations indicated.
(11) Na_2PdCl_4, $Pd(OAc)_2$, and *trans*-$Pd(NH_3)_2Cl_2$ cause visible precipitation of catalytic antibodies 38C2 and 33F12 (2.5 μM) at ≥50 μM Pd. As might be expected, the metal concentration corresponding to the onset of observable precipitation varies with antibody concentration and also varies slightly between different batches of antibody samples.
(12) Incubation of antibody 38C2 with a 2-fold molar excess of $Pd(en)Cl_2$ (relative to antibody active sites) followed by dialysis of the mixture against PBS buffer gives an antibody preparation that mediates aldol condensation at a rate identical to that of a control sample not treated with metal. Both dialyzed samples then respond to the addition of Pd^{II} with increased catalytic rates in the usual fashion.
(13) (a) Measurement of tryptophan fluorescence at 340 nm (for 280-nm excitation, see: Crowder, M. W.; Stewart, J. D.; Roberts, V. A.; Bender, C. J.; Tevelrakh, E.; Peisach, J.; Getzoff, E. D.; Baffney, B. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 5627) showed a loss of 20% of the fluorescence signal of the catalytic antibodies upon the addition of 3 equiv of Pd with respect to antibody active sites, whereas very little quenching occurs for the addition of Pd to bovine serum albumin or for the addition of K_2PtCl_4 to antibodies 38C2 and 33F12. (b) MALDI mass spectral analysis of the 33F12 F_{ab} fragment in water, in the absence and presence of Pd^{II} compounds, revealed an increase in molecular weight corresponding to 1−2 mol of metal complex

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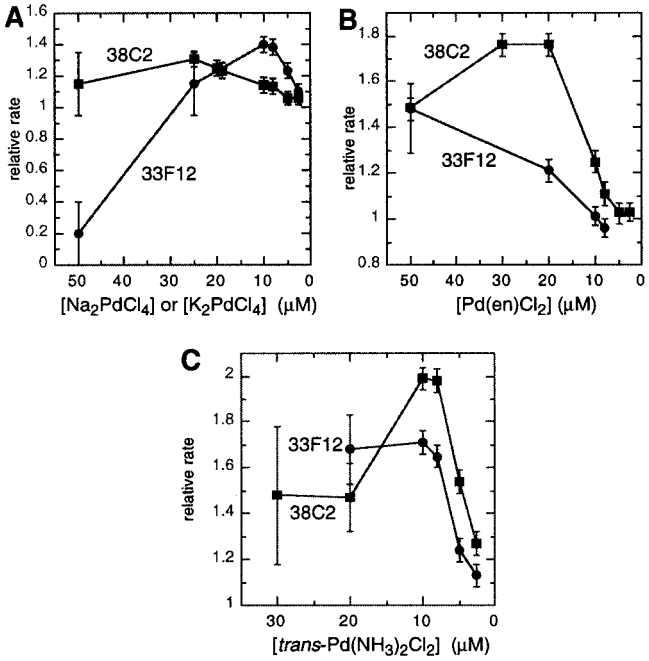

Figure 1. Rates of aldol condensation reactions of **1** + acetone in PBS buffer, in the presence of 2 μM antibody and the following additives, relative to reactions in the absence of additive: A = Na_2PdCl_4 or K_2PdCl_4; B = $Pd(en)Cl_2$; C = *trans*-$Pd(NH_3)_2Cl_2$.

Table 1. Kinetic Parameters for the Reaction of **1** with Acetone Catalyzed by Antibody 38C2 (2.6 μM) in PBS Buffer in the Presence of the Indicated Additives

additive	concn (μM)	k_{cat} (min^{-1})	K_m (μM)
none		4.3×10^{-3}	18.0
$VOSO_4$	100	3.1×10^{-3}	11.6
Na_2PdCl_4	15	6.1×10^{-3}	27.7
$Pd(en)Cl_2$	15	6.6×10^{-3}	28.4

degradation of the protein.[14] The aldol reaction is strongly inhibited by 2,4-pentanedione in both the presence and absence of metal, supporting the assumption that the active-site lysine residue is important to the catalyzed process.[3] The Fab fragment of antibody 33F12 shows the same metal-dependent behavior as the intact antibody; in no case is precipitation observed. As might therefore be expected, the greatest rate enhancement factor (2.3−2.6) is observed with this system.[7]

The enantioselectivity of the antibody-catalyzed aldol reaction is also affected by Pd^{II} additives. The two catalytically active antibodies, which exhibit similar absolute rates, substrate tolerances, and often extraordinarily high levels of asymmetric induction,[2b] give different results in the reaction of aldehyde **1** with acetone (38C2, 53% ee; 33F12, 77% ee). Furthermore, while the reaction rate increases with added Pd^{II} in both cases, the enantioselectivity improves with 38C2 but diminishes with 33F12 (Figure 2). For the reaction of acetone with substrate **2**, the 38C2-catalyzed process is accelerated, but the 33F12-catalyzed reaction is inhibited, by added Pd^{II}.[7,15]

Antibody-catalyzed aldol reactions involving the following components are all inhibited by Pd^{II} over the same concentration

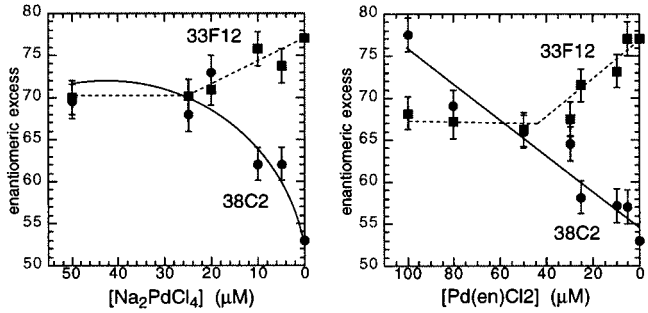

Figure 2. Enantiomeric excess (ee) vs Pd^{II} concentration for the reaction of **1** with acetone, catalyzed by the indicated antibodies at 2.0 μM. Similar results are observed at antibody concentrations of 5 and 10 μM with proportional increases in metal concentration.

range as the **1** + acetone reaction is accelerated: (1) **1** + cyclopentanone, (2) **1** + hydroxyacetone, (3) branched aldehyde **3** + acetone, and (4) the self-aldol condensation reaction of propionaldehyde catalyzed by the same antibodies.[2b,16] The antibody-catalyzed retro-aldol conversion of compound **4** into **1** + acetone was found to be inhibited by $VOSO_4$, but is accelerated only mildly by Pd^{II} compounds (relative rates $\leq$1.4 in the presence of Na_2PdCl_4 or $Pd(en)Cl_2$). A complete kinetic study of this process was not performed. Simple Pt^{II} amine complexes, which are close structural analogues to their Pd^{II} congeners,[17] exhibit a very weak form of the Pd^{II} aldol effect.[7]

Our results show that the antibody-catalyzed aldol reaction responds to Pd^{II} additives by an improvement in rate and selectivity only in a highly specific sense—for the substrate that most closely resembles the hapten to which the antibody was educated. It is unlikely that Pd^{II} binds in the active site, since one would expect much more dramatic effects on the aldol process in that event. Instead, the effect appears to be allosteric, where Pd binding induces a conformational change that promotes a closer fit of the active site to the transition state structure for the hapten-like case and a poorer fit for substrates that differ from the hapten. The technique of reactive immunization is expected to produce antibodies of a "permissive" nature, since the evolution of multiple noncovalent contacts to the hapten (constructing a "lock" for the "key") is not required to achieve the tight binding that is the goal of the immune response. The results reported here suggest that noncovalent interactions may play a limited role in the reactive immunization process, that cofactors can be found to amplify these interactions, and that such amplification may be expected to make promiscuous catalysts more specific. It may therefore be advantageous when addressing particular problems of catalysis to couple reactive immunization (or other techniques of combinatorial catalyst development) with cofactor screening.

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Supporting Information Available: Experimental procedures for kinetics and spectroscopic experiments (8 pages). See any current masthead page for ordering information and Web access instructions.

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bound per F_{ab} polypeptide when incubated with 5 equiv of Pd precursor. (c) The Pd^{II}−catalyzed ring closure of an *o*-allylphenol is inhibited by both catalytic antibodies at the micromolar level, demonstrating the ability of the antibody to sequester the metal center. See the Supporting Information for details.

(14) Palladium salts can mediate hydrolysis of oligopeptide amide bonds adjacent to the site of metal binding: Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1996**, *118*, 51.

(15) Asymmetric induction is poor for substrate **2** with both antibodies (15−25% ee) and is diminished further in each case by the addition of Pd^{II} compounds.

(16) Reactions 1−4 were not as thoroughly examined as the acceleratory reactions described above. However, the inhibitory effect of Pd^{II} is clear in each case, is proportional to metal concentration, and reaches a maximum of approximately 50−60% of the nonadditive rate at approximately 40 μM Pd^{II}.

(17) (a) Pettit, L. D.; Bezer, M. *Coord. Chem. Rev.* **1985**, *61*, 97−114. (b) Potentiometric titration of $Pd(en)Cl_2$ under conditions of high NaCl concentration very close to those used here [Tercero-Moreno, J. M.; Matilla-Hernández, A. M.; González-García, S.; Niclós-Gutiérrez, J. *Inorg. Chim. Acta* **1996**, *253*, 23] show that the major species present at micromolar total Pd levels are the following: $Pd(en)Cl_2$ (~85%), Pd(en)Cl(OH) (~12%), and $Pd(en)(Cl)(H_2O)^+$ (~3%). Indeed, $[Pd(en)(H_2O)_2]^{2+}$ and [*cis*-$Pd(NH_3)_2(H_2O)_2]^{2+}$, generated by treatment of $Pd(en)Cl_2$ and *trans*-$Pd(NH_3)_2Cl_2$ with $AgNO_3$ in H_2O, gave the same aldol response as their chloride precursors in antibody-catalyzed reactions performed in PBS buffer.