varied. Acetonitrile solutions containing the sensitizer and the quencher (either dimethylthymine[9] or its cis,syn dimer[10]) were prepared in sealed cuvettes and purged with dry nitrogen. The fluorescence intensity was measured and compared with the fluorescence of an identical solution which contained no quencher. The resulting Stern–Volmer plots[11] showed good linearity and gave correlation coefficients of 0.98 or greater. The k_q values were calculated using literature values for the sensitizer singlet lifetimes (τ).

The effect of E_{ox}^* on k_q is shown in Figure 1 and Table I. As E_{ox}^* becomes increasingly negative, k_q increases, reaching the diffusion limit near −2.7 V. Without assuming any particular quantitative model, two qualitative generalizations can be made. First, the clear correlation of k_q with E_{ox}^* is consistent with the proposed anion radical mechanism and establishes its generality.[12] A plot of k_q vs the sensitizer singlet energy, E_{00}, for example, showed no clear correlation. The latter indicates that charge transfer is the only significant quenching mechanism. Second, as the sensitizer potentials become more positive than −2.5 V (vs SCE), the rate drops off significantly. Therefore, sensitizers with potentials more positive than ca. −2.4 V would probably not be effective at initiating cleavage via the SET mechanism.

For systems which behave as eq 1, k_q can be quantitatively related to ΔG_{cl} by the Rehm–Weller relationship:[13]

$$k_q = \frac{k_{dif}}{1 + 0.25\{\exp(\Delta G_{ct}^*/RT) + \exp(\Delta G_{cl}/RT)\}} \quad (3)$$

where

$$\Delta G_{ct}^* = \left[\left(\frac{\Delta G_{ct}}{2}\right)^2 + \left(\frac{\lambda}{4}\right)^2\right]^{1/2} + \frac{\Delta G_{ct}}{2} \quad (4)$$

The data in the present work were fit to this relationship using λ (the solvent reorganization energy) and E_{red} (= $E_{ox}^* - \Delta G_{ct}/23.06$) as adjustable parameters. The values thus derived are E_{red} = −2.60 V (vs SCE), λ = 15.7 kcal/mol for dimethylthymine dimer and E_{red} = −2.21 V, λ = 13.7 kcal/mol for the monomer. To test the validity of this method, it was applied to a quencher with a known reduction potential, methyl benzoate. The value obtained by our method, −2.26 V, is in reasonable agreement with the value reported from electrochemical measurements (−2.3 V).[14]

One approximation in this analysis is the structural difference between the model compound and the biological substrate (thymidine) which is unsubstituted at N-3. (Thymine, 1-alkylated thymine, and their dimers are not sufficiently soluble for this study.) Methyl groups are weakly electron donating. Therefore, it is possible that E_{red} for the biological substrate is slightly more positive. However, a difference of more than 0.10 V would be very surprising.[15]

Electron transfer to the dimer alters the thermodynamics of the cleavage step. Analysis of a simple thermodynamic cycle gives eq 5. The extra potential required to reduce the dimer adds to the driving force of the cleavage step. The free energy change of the anion radical cleavage step (ΔG_{anion}) depends on the reduction potentials of the dimer and the monomer along with the $\Delta G_{neutral}$ (cleavage of the neutral dimer to neutral monomer):

$$\Delta G_{anion} - \Delta G_{neutral} = 23.06(E_{red}^{dimer} - E_{red}^{monomer}) \quad (5)$$

Our results show that cleavage of the anion is 9 kcal/mol more exergonic than cleavage of the neutral.[16]

The data here are consistent with the proposed reductive SET mechanism for photolyase.[17] The relevant enzymic chromophore is a 1,5-dihydroflavin. In free solution, these species have E_{ox}^* of −2.6 V.[18] Therefore the SET step in the enzymatic reaction is approximately thermoneutral. This is in agreement with the picosecond measurements of Okamura et al.[19] These workers showed that the excited-state flavin interacts with the damaged DNA very rapidly, with a rate constant of 5.5×10^9 s^{-1}. This requires that ΔG_{cl} in the enzyme be <1 kcal/mol.[20]

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Registry No. DNA photolyase, 37290-70-3; dimethylthymine, 4401-71-2; *cis,syn*-dimer, 3660-32-0.

Supplementary Material Available: A table of sensitizers, $k_q\tau$ values, and the parameters used in the data analysis (1 page). Ordering information is given on any current masthead page.

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Cross-linked Enzyme Crystals as Robust Biocatalysts

Nancy L. St. Clair and Manuel A. Navia*

Vertex Pharmaceuticals Inc., 40 Allston Street Cambridge, Massachusetts 02139-4211

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Chemical cross-linking of enzyme crystals stabilizes the crystalline lattice and its constituent enzyme molecules, forming highly concentrated immobilized enzyme particles which can be lyophilized and stored indefinitely at room temperature. Cross-linked enzyme crystals (CLCs) retain catalytic activity in harsh conditions, including temperature and pH extremes, exogenous proteases, and exposure to organic or aqueous solvents and aqueous–organic mixtures. Lyophilized CLCs can be reconstituted easily in these solvents as active, monodisperse suspensions. We present data comparing free enzyme vs CLCs of thermolysin, which is used in the manufacture of the artificial sweetener aspartame.[1,2] Results with other enzymes suggest that the CLC process may be broadly applicable.

* Author to whom correspondence should be addressed.

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Table I[a]

	% maximum activity	
	free thermolysin	CLCs of thermolysin
acetonitrile	42	102
dioxane	66	97
acetone	75	99
THF	36	96

[a] An equal volume of organic solvent was added and vortexed into either a free thermolysin solution or a CLC slurry (both at 10 mg/mL concentration) in 10 mM Tris-HCl buffer, pH 7. After 1 h of incubation at 40 °C, activity was assayed as described in Figure 1.[12] Free thermolysin concentration is limited to a maximum of 10 mg/mL by precipitation in these solvents; CLC concentration is limited only by the volume of the crystals in suspension.

Enzyme-catalyzed processes enjoy significant advantages over conventional chemical methods.[3,4] Widespread enzyme use has been limited, however, by storage and handling problems, especially enzyme denaturation in aqueous-organic solvent mixtures.[5] These problems have made it difficult to exploit solubility differences between substrates and products to improve synthetic yields.[6] In addition, many substrates and products are unstable or insoluble in aqueous media where enzyme use is favored. Crystallization has long been used as a means of protein purification.[7,8] Search strategies for the growth of large crystals[9] for X-ray diffraction will also produce microcrystals routinely[8]—though these are seldom reported. Microcrystals ($\sim 10^{-1}$ mm in size) are preferred for CLCs because their small cross section minimizes substrate-product diffusion problems.[10] In 1964, Quiocho and Richards proposed glutaraldehyde cross-linking to improve crystal stability in diffraction experiments.[11] Subsequently, cross-linked carboxypeptidase-A crystals were shown to retain catalytic activity in aqueous solution.[10] We now demonstrate near-maximum catalytic activity for cross-linked microcrystals of thermolysin in a broad range of conditions otherwise incompatible with enzyme function.

Stability of Thermolysin CLCs. Following crystallization, cross-linking, and lyophilization, rehydrated thermolysin CLCs retain 80% of the activity of free thermolysin (Figure 1).[12] Remarkably, thermolysin CLCs are stable in aqueous-organic solvent mixtures where enzymes usually denature; after 1 h of incubation in 50% aqueous tetrahydrofuran at 40 °C, free thermolysin retains only 36% of its activity, while thermolysin CLCs retain >95% activity in all of the aqueous-organic solvents tested (Table I).[12] Because of crystal uniformity, lyophilized CLCs can be reconstituted by simple addition of solvent and remain monodisperse (even in near-anhydrous organics), unlike lyophilized free enzyme. CLCs also retain full activity at pH extremes and at elevated temperatures, even after 4 days of continuous incubation at 65 °C (Figure 1a).[12] Thermolysin CLCs are resistant to degradation in high concentrations of exogenous proteases such as Pronase (a mixture of *Streptomyces griseus* proteases[13] capable of digesting most proteins) after incubation for 4 days; free thermolysin is digested in 90 min (Figure 1b).[12] Protease resistance is shared by porcine liver esterase, porcine pancreatic elastase, and all CLCs investigated so far.[12,14]

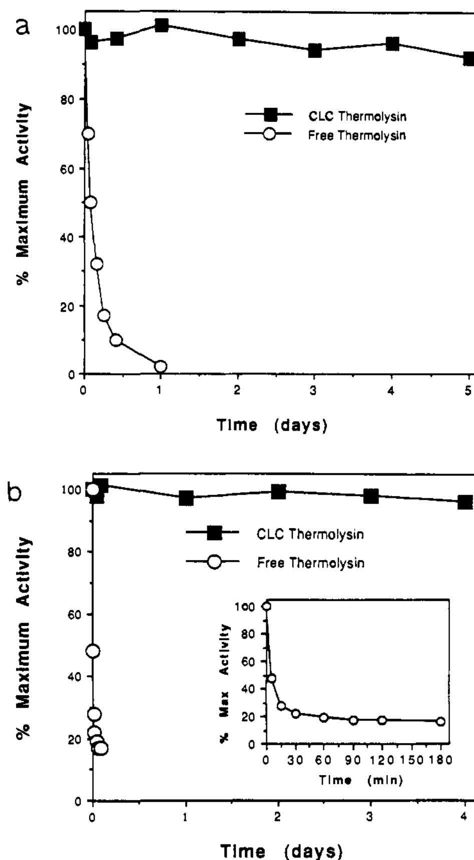

Figure 1. Maximum activity (%) of CLC and free thermolysin. Thermolysin microcrystals form on adding nine volumes of demineralized water to one of concentrated (100 mg/mL) crude thermolysin in 1.0 M calcium acetate, 30% dimethyl sulfoxide, 50 mM Tris, pH 7.0.[12] Crystals were cross-linked in 12.5% glutaraldehyde[25] and 50 mM Tris, pH 6.5, for 1 h. Washed CLCs were lyophilized as a crystalline slurry in deionized water, pH 7.0.[26] CLC and free thermolysin catalytic activity was assayed by hydrolysis of furylacryloylglycyl-L-leucine amide (Schweizerhall).[12,27] Absorbance at 345 nm was fit to a pseudo-first-order rate equation[28] to determine k_{cat}/K_m, which is 3.7×10^4 M^{-1} s^{-1} for free thermolysin and 3.0×10^4 M^{-1} s^{-1} for CLCs. (a) Thermolysin CLCs retain activity after prolonged exposure at 65 °C.[12] The activity of CLC and soluble thermolysin was assayed at room temperature following incubation in 10 mM calcium acetate and 50 mM Tris, pH 7.0, buffer at 65 °C. Total protein concentration was 10 mg/mL. At the times indicated, aliquots were removed from each reaction mix, and thermolysin activity was assayed spectrophotometrically as described above. (b) Thermolysin CLCs are resistant to exogenous proteolysis.[12] Free and CLC thermolysin were incubated in 50 mM Tris, pH 7.5, buffer at 40 °C in the presence of the enzyme Pronase (Calbiochem). The Pronase to thermolysin ratio was 1:40. At the times indicated, aliquots were removed from each reaction mix, and thermolysin activity was assayed spectrophotometrically as described above. Inset: Detail of soluble thermolysin time course.

Discussion. The remarkable temperature, pH, and solvent stability observed with thermolysin CLCs may result from restricted access to required kinetic intermediates on the denaturation pathway,[15] as a consequence of crystal packing reenforced by covalent cross-links. Noncrystalline lyophilized enzyme suspensions in near-anhydrous organic solvents also demonstrate stability,[4,16] even at high temperatures,[17] due in part to the inac-

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cessibility of water-dependent denaturation intermediates. As such, minimal added water in these systems can lead to rapid loss of activity, unlike CLCs which are stable at all water concentrations. Proteolysis resistance of CLCs is explained by the exclusion of exogenous protease, due to the size of the solvent channels defined by the crystal lattice. Large substrate/product molecules (e.g., proteins, polynucleotides, etc.) will also be restricted, of course, though most smaller substrates can be accommodated.

Conventional immobilized enzymes are principally used to facilitate catalyst recovery,[18] but they can also provide modest stability enhancements.[19] Formulated as beads or particles, however, these systems consist mostly of inert carrier material. CLCs provide their own support, and so achieve enzyme concentrations close to the theoretical packing limit—in excess of even highly concentrated enzyme solutions. As such, CLCs are particularly attractive in biosensor applications, where the largest possible signal per unit volume is often critical. We have formulated cross-linked enzyme crystals of jack-bean urease for use in clinical biosensor applications to measure urea levels in the circulation as an early indicator of renal disease.[20]

Manufacturing applications can be particularly difficult for enzyme catalysts. The artificial sweetener aspartame (L-aspartate-L-phenylalanine methyl ester), for example, can be readily obtained by deprotection of a precursor species that is formed by the thermolysin-catalyzed regio- and stereoselective condensation of (benzyloxycarbonyl)-L-aspartic acid and DL-phenylalanine methyl ester in a nonaqueous solvent.[1,2,21] Thermolysin was found, by trial and error, to be a suitable, readily available enzyme for use in this process.[1] Chemical synthesis,[22] however, has proven more viable for the large-scale manufacture of aspartame, due in part to unsatisfactory enzyme stability over the long term. We have adapted the enzymatic process[21] to take advantage of the enhanced solvent and temperature stability described above for thermolysin CLCs and have been able to produce the aspartame precursor in buffer-saturated ethyl acetate at 55 °C over a period of 18 days without significant loss of enzyme stability. Under these conditions, free thermolysin was found to be inactive on the fourth day.[20]

Temperature and protease resistance also make CLCs useful in therapeutics. Suitably implanted or orally administered CLCs may provide an alternative to gene replacement therapy for the correction of some inherited enzyme deficiencies. Finally, diffraction-quality FAB fragment crystals are known for many classes of antibodies.[23] Catalytic antibody[24] CLCs might enable practical chemical synthesis using these molecules, accelerating the benefits promised by this technology.

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Registry No. Aspartame, 22839-47-0; *N*-[*N*-[(phenylmethoxy)-carbonyl]-L-α-aspartyl]-DL-phenylalanine methyl ester, 110220-25-2.

Supplementary Material Available: Experimental details for the thermolysin CLCs and plots of the absorbance and activity of CLCs and free enzyme (11 pages). Ordering information is given on any current masthead page.

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β-Hydride Elimination for an Amine Ligand and the Microscopic Reverse: The First Report of a *cis*-Iminium Hydride in Equilibrium with Its Amine Precursor

Joseph Barrera, Stephen D. Orth, and W. Dean Harman*,†

Department of Chemistry, University of Virginia
Charlottesville, Virginia 22901

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β-Hydride elimination of metal alkyl complexes and the reverse process of olefin insertion into a metal-hydride bond are fundamental transformations in organometallic chemistry.[1] In particular, the transfer of a β-hydrogen atom from an alkyl ligand to the metal through a four-center transition state is the primary mechanism for metal alkyl decomposition. Ubiquitous in coordination complexes, aliphatic amine ligands are isoelectronic to alkyl ligands and would be expected to undergo the analogous reaction under appropriate conditions. Although β-hydride elimination is commonly invoked as the mechanism for the decomposition of amide complexes to metal hydrides,[2] the direct transformation of a coordinated amine to an iminium hydride species has never been observed. We wish to report such a transformation on Os(II), along with the observation of the microscopic reverse: iminium insertion into a metal-hydride bond.

Using a modified procedure originally outlined by Magnuson et al. for the synthesis of (dinitrogen)tetraammineosmium(II) complexes,[3] the compound *cis*-$[Os(NH_3)_4(NH_2Pr)(N_2)](OTf)_2$ (**1**, Pr = propyl, OTf = $CF_3SO_3^-$) was obtained from $[Os(NH_3)_4(N_2)_2](OTf)_2$ by refluxing the latter in a 1,2-dimethoxyethane (DME) solution of propylamine.[4,5] The dinitrogen ligand of **1** was then removed by metal oxidation in a triflic acid/bromine solution to produce $[Os(NH_3)_4(NH_2Pr)(OTf)](OTf)_2$ (**2**).[5]

† Camille and Henry Dreyfus Teacher-Scholar, 1992.

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