Investigations of an Antibody Ligase

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Abstract: Further investigation of the monoclonal antibody 16G3 has revealed that it not only couples activated amino acids to form dipeptides with high turnover rates but also couples an activated amino acid with a dipeptide to form a tripeptide, as well as an activated dipeptide with another dipeptide to give a tetrapeptide. The catalytic rates for these reactions greatly exceed the background rate of ester hydrolysis providing average yields of 80% within the assay time of 20 min. Importantly, the amount of product inhibition is low, allowing for high yields of products using multiple addition of substrates to the same antibody reaction mixture. A sequential mechanism is employed by 16G3 for dipeptide coupling, and this mechanism appears to hold for the formation of the other peptides. High catalytic selectivity is observed for the nucleophilic α-amino group of an α,*β*-diamino nucleophile and for the para substituent on the activated ester, traits that are consistent with hapten design. The former chemoselectivity is crucial for the condensation of fragments which are unprotected at the *ϵ*-amino group of lysine.

Introduction

In the past few years significant strides have been made in the pursuit of synthesizing unique proteins or peptides *in vitro*. At this time, peptides containing approximately 50 amino acids can be synthesized efficiently in a stepwise manner using solid-support methodology or solution chemistry-based fragment condensation. Larger peptides have also been synthesized both in solution[1a] or on solid support.[1b] Because many proteins are larger than these, other methods are needed to couple fragments. One approach that has been employed in the construction of the transcription factor-related protein cMyc-Max[2a] and a HIV-1 protease analog[2b] entails the stepwise synthesis of peptide fragments of about 30−50 amino acids in length and then their chemoselective ligation. Solid-phase synthesis was employed to incorporate the unique active groups that provided for the thioester and oxime linkage in the former study and the thioester and disulfide bond in the latter. These syntheses did not require protecting groups, and the proteins were active. Thus, coupling occurred in positions that reconstructed the proteins with the correct orientation and did not interfere with their function. An oxime linkage has also been used to form a cyclic peptide,[3] and disulfide bonds were used in the partial construction of the protein basic pancreatic trypsin inhibitor.[4]

Other methods for coupling are based on combining native or nonnative peptide fragments using enzymes or antibodies. Subtilisin has been modified by Wells et al.[5] by a double mutation to give an effective ligase without extensive protease or esterase activity The resulting subtiligase binds seven amino acids in its active site with the first, fourth, and fifth amino acid from the *N*-terminus providing most of the binding energy. A broad range of specificity was observed with some amino acids being well recognized while others are recognized only weakly, thereby limiting the choice of amino acid for chemical reaction. So far, unnatural amino acids have not been shown to be recognized by subtiligase, but in the synthesis of ribonuclease A,[6] Wells successfully incorporated unnatural amino acids within peptide fragments that had been prepared by solid-phase synthesis.

Catalytic antibodies are an attractive choice for peptide coupling considering that peptide fragments are a natural epitope and antibodies have been constructed to catalyze a wide range of chemical reactions.[7] X-ray crystallographic analyses[8] reveal that antibodies bind peptides of various lengths in elongated grooves using van der Waal, hydrogen bonding, and ionic contacts for recognition. Therefore, antibodies can either be used for highly specific coupling or be designed to recognize a variety of natural and unnatural amino acids. As a first step toward generating antibodies capable of coupling unprotected

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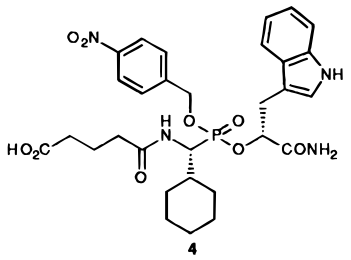

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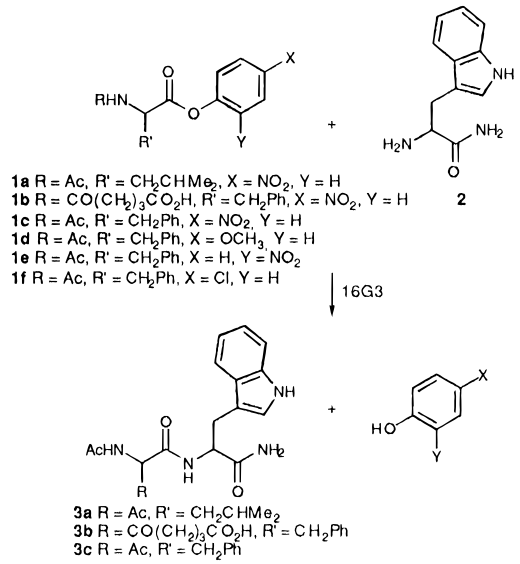
Figure 1. Hapten (**4**) used to elicit antibody 16G3.

Scheme 1



peptide fragments, we generated antibody 16G3 which catalyzes the formation of a di-, tri-, and tetrapeptide *(vide infra)*.

Results and Discussion

In a recent publication,[9] we demonstrated that antibody 16G3 was able to catalyze the reaction between **2** and various activated esters including **1a**,**c** (Scheme 1) forming a series of dipeptides. Product formation was accelerated by approximately 400-fold over background for the coupling of activated *N*-acetyl-L-leucine (**1a**) with D-tryptophan amide (**2**). 16G3 was obtained by using hapten (**4**) (Figure 1) as an immunogen,[10] and even though only one diastereomer was used, collectively the antibodies react with all stereomeric combinations of ester and amide. The smaller size of the substrates compared to the hapten is one possible explanation for the lack of chiral recognition observed in the coupling reaction.[9] A lack of preference is also observed in hapten binding where 16G3 binds both diastereomers of **4** with K_D values less than 2 nM. It is noteworthy, however, that antibody 16G3 does not catalyze the racemization of N-acylated *p*-nitrophenyl esters such as **1c**.

To determine the mechanism employed by 16G3, the reaction of **1b** with **2** was examined in detail under steady-state conditions. The change in velocity of product formation was measured with the concentration of substrate **1b** held constant while varying the concentration of **2**. The converse experiment was also performed; the resulting plots (Figure 2) reveal a sequential mechanism with the binding of one substrate not affecting the binding of the second substrate. Second-order plots gave Michaelis constants of 100 μM for **1b** and 900 μM for **2** and a k_{cat} of 0.6 min^{-1}/antibody binding site. An approximate

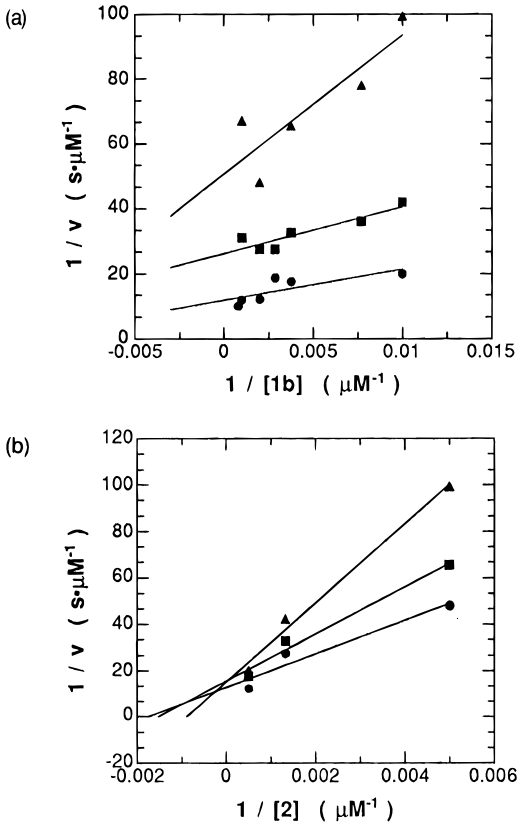


Figure 2. Lineweaver−Burke plots for 16G3 catalysis with (a) **2** held at constant concentrations of 200 μM (▲), 750 μM (■), and 2000 μM (●) reacting with **1b** from 100 to 1000 μM (▲), 100 to 1000 μM (■), and 100 to 1270 μM (●) and (b) **1b** held at constant concentrations of 100 μM (▲), 266 μM (■), and 500 μM (●) reacting with **2** from 200 to 2000 μM (▲, ■, and ●).

value for the background velocity of dipeptide formation is 0.04 M^{-1}•min^{-1}; comparing k_{cat}•K_m^{-1} for the weaker binding (less favorable) substrate **2** (7.0×10^2 M^{-1}•min^{-1}) gives a rate enhancement of 2×10^4. Access to a larger range of substrate concentrations was limited by the poor solubility of **1b**.

Bolstered by the successful coupling of amino acids, the potential of 16G3 was tested further by the formation of larger peptides. The activated esters of *N*-acetyl-L-phenylalanine (**1c**) and *N*-acetyl-glycyl-L-phenylalanine (**7**) were allowed to react with D-tryptophanyl-glycine (**5**) to produce the N-acetylated tripeptide **6** and N-acetylated tetrapeptide **8**, respectively (Schemes 2 and 3). Formation of **6** and **8** was extremely facile with almost complete conversion (92% tripeptide formation and 70% tetrapeptide formation) within the assay time of 20 min. Rigorous kinetic analyses were not performed, but a lower limit for product formation is 50 μM•min^{-1} for **6** and 30 μM•min^{-1} for **8** with background velocities for both of 0.2 μM•min^{-1}. These velocities for 16G3 are a lower estimate, considering that the values are obtained at 30 s into the assay (the first data point that could be accurately taken) and that the poor solubility of substrates **1c** and **7** prevented substrate saturation of the antibody active site.

The catalytic efficiency of 16G3 is a result of *both* its rate enhancement for bond formation and the lack of substantial product inhibition. The high turnover of 16G3 was first evident with the production of 1.8 mM dipeptide **3a** in approximately 2 h using 20 μM antibody. A similar production curve was obtained for **8** with 0.2 mM being synthesized in 2 h also using 20 μM 16G3 (Figure 3a). To obtain estimates for the bond formation rates and product inhibition constants, the time courses for multiple addition of substrates to a single antibody solution

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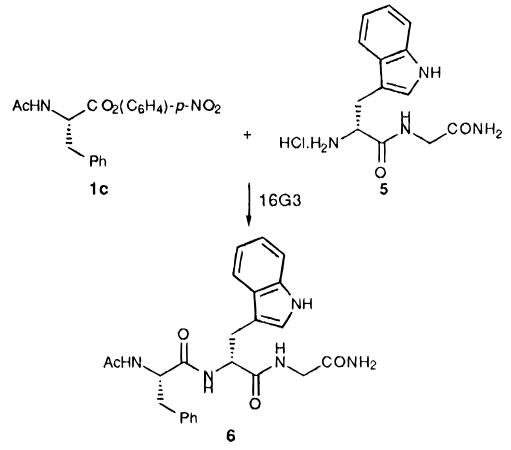
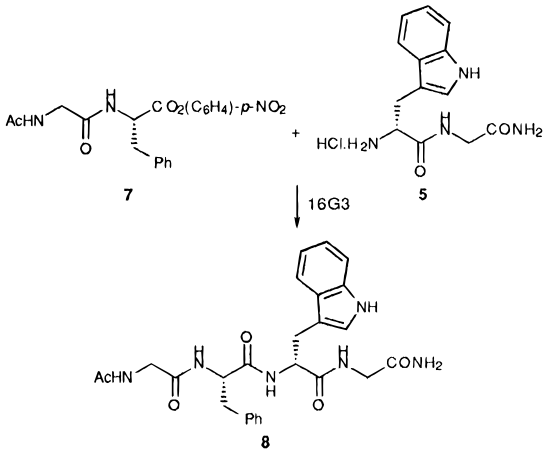
Scheme 2



Scheme 3



for the reactions producing the di- and tetrapeptides (Figure 3a) and *p*-nitrophenol (Figure 3b), along with their product versus time curves, were simulated by the KINSIM computer program using a simple kinetic mechanism (Scheme 4). Also simulated was the time course for the tripeptide reaction (time course not shown). It was assumed that the antibody establishes a rapid equilibrium with the substrates prior to catalysis and that bond formation is the rate-determining step, k_{cat}. For simulations, major variations were performed on the rates of bond formation and product dissociation constants.

By examining the estimated rate and binding constants (Table 1), it seems that the catalytic mechanism employed by 16G3 is the same for all substrates because the corresponding individual steps have similar values. Relative to the K_m value for *N*-acetyl-L-valine ester ($K_m = 4$ mM)[9], the data also show that substrate binding is more favorable for ones having larger hydrophobic substituents at the α-position (*N*-acetyl-L-leucine $K_m = 200$ μM, *N*-acetyl-L-phenylalanine $K_m = 150$ μM). This is reasonable considering that the hapten has a cyclohexyl ring at that position. When the peptide is extended there is only a small effect on binding, either as a substrate (K_m for **1a**,**c** and **7** and for **2** and **5**) or product (K_D for **3a**, **6**, and **8**). Thus, the smaller amount of tetrapeptide produced over time compared to dipeptide is a result of the poorer water solubility of **7** (its maximum concentration is below its K_m value) compared to **1a**. For larger peptides, the amount of specific binding and the rate of bond formation should be roughly constant because only portions of the substrates, roughly the size and shape of the hapten, interact with the antibody pocket. Other binding energies can result from nonspecific interactions between the polypeptide and the antibody.

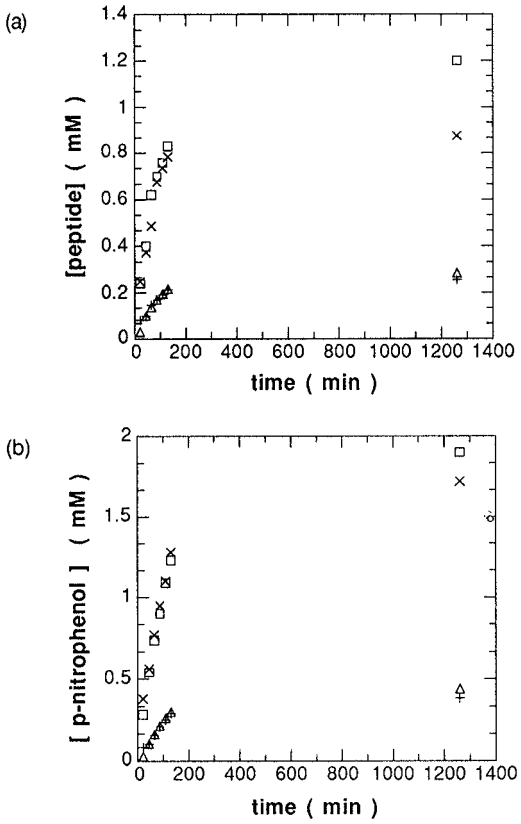


Figure 3. (a) Amount of dipeptide **3a** (□) (values are multiplied by 0.5) and tetrapeptide **8** (△) formed with time using 20 μM 16G3. Also given are the calculated amounts of **3a** (×) (0.5×) and **8** (+) derived from KINSIM analysis using the constants given in Table 1. (b) Amount of *p*-nitrophenol produced in the reaction forming the dipeptide (□) (0.5×) and tetrapeptide (△) and their corresponding values derived from KINSIM analysis (×) and (+), respectively.

Scheme 4

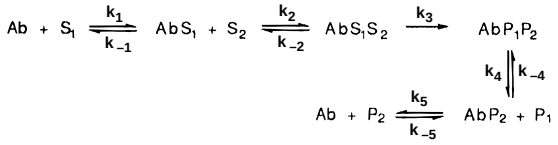
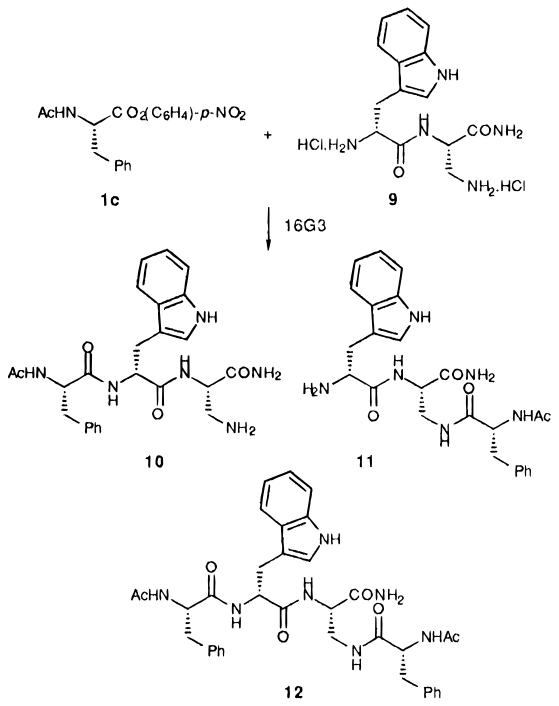


Table 1. Kinetic and Binding Constants Derived from KINSIM Analysis for the Mechanism Displayed in Scheme 4 That Describes the Reaction between the Various Substrates and 16G3

reaction	k_{cat} (s^{-1})	product	K_D (μM)	reactant	K_m (μM)
1a + **2**	0.05	**3a**	170	**1a**	200
1c + **5**	0.02	**6**	140	**1c**	150
7 + **5**	0.01	**8**	100	**2**	870
		p-$O_2NC_6H_5OH$	330	**5**	900
				7	120

For catalytic antibodies to be useful in the condensation of fragments unprotected at the ϵ-amino groups of lysine, it is critical that the antibodies catalyze acylation only at the α-amino group of the nucleophile. This requirement was tested by measuring the product distribution of a reaction between the bis amino substrate **9** with **1c** (Scheme 5). In the absence of 16G3, the reaction afforded **10** and **11** in approximately equal amounts with a velocity of 0.1 μM·min^{-1}. But in the presence of 16G3, the desired reaction proceeded with greater than 80-fold preference for **1c** reacting with the α- relative to the β-amino group based on initial velocity measurements (i.e., 8 μM·min^{-1} for **10** and 0.1 μM·min^{-1} for **11**). Importantly, the production rate of **11** did not change in the presence of 16G3,

Scheme 5



and **12** was not formed with or without 16G3 (the reactions were run with excess **9**).

Specificity for the electrophilic species is also high. Within the ester series, the *p*-NO_2 substrates **1a**−**c** are turned over by the antibody, but the reactions involving the *p*-OCH_3 (**1d**), *m*-NO_2 (**1e**), and *p*-Cl (**1f**) substrates are not catalyzed by 16G3. Even though the spontaneous hydrolysis of **1e** limited its reaction time, it was determined that **1e** does not bind strongly to the antibody pocket because it was not an inhibitor of the reaction between **1b** and **2**.[11] It is evident that the *p*-NO_2 moiety on the substrate is crucial for both binding and activation. More importantly, having the substrate contain a *p*-nitrophenyl ester, whereas the antibody was induced by a hapten containing a *p*-nitrobenzyl in the corresponding position, appears to provide reduced product inhibition by *p*-nitrophenol (K_D = 330 μM, Table 1) and subsequently higher yields of product. Evidence for this hypothesis can be obtained by comparing the turnover number for *p*-nitrophenyl ester hydrolysis by the antibody 43C9[12] with 16G3 catalysis. 43C9 was raised against a hapten containing a *p*-nitrophenyl substituent, and its catalysis was limited by product inhibition of *p*-nitrophenol (K_D = 1 μM).

Binding of a nitrophenyl ester in a pocket sculpted for a nitrobenzyl moiety was anticipated to affect the catalytic steps depicted in Scheme 4. Binding should be weaker (especially for substrates with small side chains) because optimal binding of the nitro group exposes the phenolic oxygen atom to a hydrophobic patch on the antibody surface reserved for the methylene group. Binding of the transition state should also be weaker due to improper alignment of the atoms, but this loss in stabilization, which reduces the rate of bond cleavage, could be countered by the less stable nitrophenyl ester. Finally, once bond cleavage occurs, *p*-nitrophenolate will be driven from the binding pocket as a result of its greater negative charge. The increased electron density should interact more unfavorably with the hydrophobic patch and thereby be partially transferred by resonance through the aromatic ring onto the nitro group which is also destabilizing.

One advantage of using catalytic antibodies is that they readily form a chiral binding pocket when challenged with a chiral hapten. In a recent review,[13] Kirby's data accumulation on catalytic antibodies showed they have a great potential for asymmetric catalysis. Considering that hapten (**4**) contains three stereogenic centers, it is surprising that the antibodies so far isolated from the immune repertoire do not discriminate among the different enantiomers of the substrates. On the basis of the rates for different activated esters[9] and the greater affinity for esters containing larger side chains, we postulate that the loss of chiral recognition for 16G3 could also be due in part to the mismatch between the combining site induced by the *p*-nitrobenzyl hapten and the *p*-nitrophenyl substrate. If one assumes that the para nitro-substituted aromatic ring provides the strongest binding epitope for the substrate, binding of the substrate will result in a shift of the ester, by approximately the one methylene unit away from the pocket designed for the side chain, and thus a loss of chiral recognition.

Results from the study of antibody 9B5.1,[14] which also catalyzes peptide bond formation, corroborate our hypothesis for the weaker chiral recognition observed with 16G3. 9B5.1 was raised against a hapten containing a phenyl ester and designed to catalyze peptide bond formation between a phenylalanyl amino group and a phenyl ester derivative of L-alanine. Since the phenyl ester substrate was not reactive, the ester was converted to an acyl azide giving a reactive substrate with a $k_{cat}{\cdot}K_m^{-1}$ value of 3.9×10^3 $M^{-1}{\cdot}min^{-1}$and a rate enhancement of 10^2 derived from the relationship $k_{cat}{\cdot}K_m^{-1}{\cdot}k_{uncat}^{-1}$. It should be noted that calculations of rate enhancements using these ratios for bimolecular reactions require the lower of the two $k_{cat}{\cdot}K_m^{-1}$ values found for the two substrates. The flux through the catalyst is limited by the least favorably processed substrate, and thus, the efficiency of antibody 9B5.1 is 10^2 rather than the reported 10^4.[14] More importantly, changing the acylating agent to a nonrecognizable epitope, an azide, did not substantially alter the acyl donors orientation in the binding pocket which is evident by its small Michaelis constant (K_m = 15 μM). Thus, the methyl side chain of the acyl azide still had access to the binding pocket, resulting in a 7.3-fold enhanced rate for the L- versus D-alanine-derived azide. This level of chiral recognition is on the same scale as observed for *N*-acetyl-L- versus *N*-acetyl-D-phenylalanine with 16G3.

Accordingly, greater chiral recognition should occur for substrates containing larger side chains that can enter the hydrophobic pocket. This is indeed true and evident by examining the change in effective molarity of *N*-acetyl-L- versus *N*-acetyl-D-valine (45 to 26 M) compared to *N*-acetyl-L- versus *N*-acetyl-D-phenylalanine (550 to 71 M).[15] As the side chain substituent becomes larger, chiral recognition increases from 57% to 87% along with a 10-fold rate enhancement. Another means for enhancing chiral recognition would be to use a *p*-nitrobenzyl ester as a substrate, but this ester is less reactive and not turned over by the antibody. In fact, 16G3 was originally designed[9] to catalyze a variety of reactions via the cyclohexyl ring in **4**. The weaker chiral recognition, albeit fortuitous, is also of benefit as it expands the repertoire of reaction partners. Having a single antibody capable of multiple fragment couplings would save time and effort by eliminating

(11) The inhibition study was run with equal concentrations of **1e**,**b** (100 μM) and 2 mM **2**. For the experimental procedure see HPLC analysis under Steady-State Kinetics in the Experimental Section.

(12) Gibbs, R. A.; Benkovic, P. A.; Janda, K. D.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1992**, *114*, 3528−3534.

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(15) The effective molarities were derived from rates given in Table 1 of ref 9.

the need for the production of unique antibodies for each set of coupling partners.

The rate enhancement provided by 16G3 of 2×10^4 is consistent with the values obtained from the limited number of bimolecular reactions catalyzed by antibodies[16] but approximately 10-fold slower than observed for substiligase.[5a] The reaction efficiencies for both ligases are generally around 70% or greater, although subtiligase substrates are longer peptides and cyclizations catalyzed by this enzyme are also constrained to large peptide fragments. To date, 16G3 has not been challenged with larger peptides; however, we do expect that cyclization of small peptide fragments may be possible. We also anticipate that the catalytic rate may be improved because, as in the case of most antibodies, the conversion of binding energy to catalysis is not complete. For example, incorporation of additional amino acids into the hapten may enhance both binding and selectivity.

One can measure the catalytic efficiency by comparing the difference in the binding energy of hapten to substrate with the ratio of the catalyzed versus the uncatalyzed rate: $(K_{m1}\cdot K_{m2})\cdot K_i^{-1} \approx k_{cat}\cdot k_{uncat}^{-1}$.[16] For haptens that are competitive inhibitors, K_D can substitute for K_i. According to this relationship, the antibody is not a very efficient catalyst for the reaction between **2** and the *p*-nitrophenyl esters of *N*-acetyl-L-valine and *N*-acetyl-L-phenylalanine giving 5% and 17% of the maximum rate enhancement, respectively. On the other hand, the differences in the values are consistent with hapten design, where the isopropyl side chain of valine binds weaker than the phenyl side chain of phenylalanine. Even with improved technology for developing catalytic antibodies, the efficiency for catalysis may never approach 100% especially for stable acylating agents. It is possible that antibodies may require active substrates, acting only to guide more energetic species along a desired reaction coordinate.

Preparative Experiments

The requisite substrates and all possible peptidyl products required for HPLC analysis of the catalytic antibody reaction mixtures and subsequent product identification are described in detail in the Supporting Information. The synthesis of the hapten was reported previously.[10]

Conclusion

We have demonstrated that antibody 16G3 is capable of catalyzing the coupling of an amino acid with a dipeptide and the coupling of two dipeptides to form tri- and tetrapeptides, respectively, even though the hapten was originally designed to create antibodies that only catalyze the production of dipeptides. This result, along with the demonstrated rate enhancements, modest product inhibition, and critical chemoselectivity, provides impetus to producing the next generation of antibodies capable of catalyzing the ligation of large, unprotected peptides.

Experimental Section

Determination of K_D by Fluorescence Quenching. The quenching of the intrinsic antibody fluorescence caused by hapten addition was monitored using a SLM 8000 fluorimeter. Inner filtering effects were corrected for by subtracting the fluorescent change observed by the addition of hapten to a tryptophan solution of known concentration. The resulting plots were then fit, by a procedure described previously,[17] to give dissociation constants.

Steady-State Kinetics. The initial velocity of product formation was determined by measuring the change in absorbance of *p*-nitrophenol at 400 nM ($\epsilon = 9600\ cm^{-1}\cdot M^{-1}$) using a Cary (Olis-14) spectrophotometer. The appropriate amounts of substrates and antibody were mixed at 25 °C in 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.0, μ = 0.1 M NaCl, and containing 5% DMSO. After correcting for the background rate of ester hydrolysis, the k_{cat} and K_m values were derived by fitting the data to the Michaelis−Menten equation. The simulations of kinetic data were done with the program KINSIM (Barshop et al.) as modified by Anderson.[18]

The amount of product formed during a reaction was also obtained by mixing the reagents (same solvents and buffer as previously discussed), removing aliquots, quenching with perchloric acid to pH ~2.5, and separating by HPLC (Water 600E) equipped with an analytical C_{18} reversed-phase column (VYDAC, Hesperia, CA) with an acetonitrile−water:0.1% trifluoroacetic acid gradient. Products were identified by matching retention times with authentic samples, and concentrations were derived by integrating the peaks.

Antibody-Catalyzed Product Formation. The total amount of product that can be formed by 16G3 catalysis was determined by mixing 20 μM 16G3 with 2 mM **5** and either 0.1 mM **1c** or **7** at 25 °C in 0.25 mL of MOPS (pH 7.0, μ = 0.1 M NaCl, 5% DMSO). At 20 min intervals, 0.03 mL aliquots were removed and analyzed for the amount of product formed. Because the substrates are almost completely depleted at 20 min, appropriate amounts of the substrates were added to the antibody and product mixture at this time to return their concentrations back to the initial conditions. The substrate aliquots were withdrawn from very concentrated stock solutions so as to maintain the antibody concentration, giving a final concentration of 16 μM for 16G3 after six additions. In other multiple-addition experiments, the tripeptide and tetrapeptide fractions were collected, and their authenticity was confirmed by mass spectrometry.

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Supporting Information Available: Complete experimental details for compounds **1c**−**f**, **5**−**12**, **14**−**17**, **19**, **20**, and **22**−**28** (18 pages). See any current masthead page for ordering information and Internet access instructions.

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