Catalytic Antibodies with Peptidyl−Prolyl Cis−Trans Isomerase Activity

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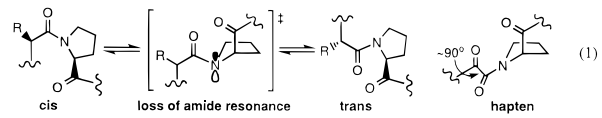
Received December 15, 1995

The peptidyl−prolyl isomerases (EC 5.2.1.8) comprise an extremely efficient ubiquitous family of enzymes that catalyze rotation about the P_1−proline amide bond. The cyclophilins and FKBPs have been identified and characterized as two distinct classes of such proteins implicated to play isomerase-dependent and -independent roles in protein folding and immunoregulation, respectively.[1−3] Although the nature of catalysis remains to be completely elucidated, it appears that distortion-related phenomena are primary components in the mechanism of action.[4]

Catalytic antibodies can be useful tools for the investigation of contributions to catalysis observed in enzymes.[5] Hapten design and substrate attenuation make it possible to program and explore active sites that model a subset of features perhaps used by the natural catalyst. The hapten **1** was anticipated to elicit antibody combining sites that were complementary to the α-ketoamide functionality and of hydrophobic character.[6]

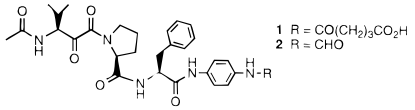
The dicarbonyl moiety in FK506 and less complex pyruvylamides is known to adopt an orthogonal conformation and possibly serve as a twisted-amide mimic.[7,8] Furthermore, isomerization of the proline amide bond is facilitated in nonpolar solvents.[9] Consequently, it was hypothesized that geometric and desolvation effects might provide sufficient energetics to increase to rate of amide rotamerization upon antibody binding of appropriate substrates (Scheme 1).

Scheme 1



(1)

A panel of 28 monoclonal antibodies (mAbs) was derived from immunization using **1** coupled to a carrier protein.[10] These mAbs were screened for catalytic activity using both fluorescence and chymotrypsin-coupled assays invoking the solvent-induced perturbation of equilibrium methodology devised by Rich and co-workers.[11] Two mAbs, VTT1E3 and VTT35C8, were found to accelerate the rate of cis to trans isomerization of the fluorophoric tripeptides **3** and **4** and the *p*-nitroanilides **5** and **6**, respectively (Table 1). These antibodies did not show cross reactivity between the substrate sets. The apparent first-order rate constants and kinetic parameters for both the uncatalyzed and antibody-mediated reactions were extracted from progress curves (Table 1, Figure 1). Use of the hapten-congruent compound **2** for binding studies and as a competitive inhibitor showed catalysis occurred specifically at the antibody active site ($K_d = 3\ \mu M$, $K_i = 10\ \mu M$).[12] Interestingly, product inhibition, tantamount to binding of the trans isomer, did not appear to be significant.[13] Finally, the reaction was not sensitive to pH variation in the physiological range, and no solvent deuterium isotope effect was observed. This is similar to the enzymatic behavior.[4]



The data implicate a distortion mechanism wherein removal of the tripeptide from an aqueous environment into the nonpolar cavity of the antibody and subsequent stabilization of a twisted intermediate promote prolyl−amide rotation. In general, antibody binding sites are hydrophobic in nature, and the association of antibody and hapten-like molecules is facilitated by classical

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(6) Since **1** positions the linker distal to the dicarbonyl, presentation of the molecule during the immune response should favor recognition of this region. This would include the hydrophobic valyl residue. It should be noted that the important factor might be not the bulk dielectric constant of the binding site, but the interactions of specific amino acids/side chains that provide a contact surface with the hapten/substrate. See: Mian, I. S.; Bradwell, A. R.; Olson, A. J. *J. Mol. Biol.* **1991**, *217*, 133−151.

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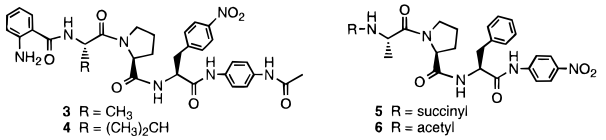
(12) The K_d and K_i values were determined by quenching of antibody fluorescence and the kinetic fluorescence assay, respectively.

(13) Using the fluorescence assay, the addition of 20 mol % of peptide equilibrated in buffer did not affect the rate. Also, competition ELISA experiments using **4** and **6** versus the immobilized bovine serum albumin conjugate of **1** showed that neither VTT35C8 nor VTT1E3 bound the trans isomer of these peptides (100 μM). In fact, only 8 of the 28 mAbs gave significant binding under these conditions where the trans isomer constituents 90% of the isomer population. For the preferential binding of the cis isomer in cyclophilin−substrate complexes, (a) Ke, H.; Mayrose, D.; Cao, W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3324−3328. (b) Kallen, J.; Walkinshaw, M. D. *FEBS Lett.* **1992**, *300*, 286−290. (c) Kallen, J.; Spitzfaden, C.; Zurini, M. G. M.; Wider, G.; Widmer, H.; Wüthrich, K.; Walkinshaw, M. D. *Nature* **1991**, *353*, 276−279.

S0002-7863(95)04206-5 CCC: $12.00

Table 1. Kinetic Constants for VTT1E3 and VTT35C8 Substrates[a]



compd[b]	k_{uncat} (s^{-1}) × 10^3	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1}) × 10^2	k_{cat}/k_{uncat}
3	4.1	1100	11	27
4	3.0	400	4.0	13
5	4.5	680	6.8	15
6	4.9	170	1.7	3.4

[a] Determined at 4 °C in 100 mM EPPS, pH 8.0 with 2.5% trifluoroethanol (TFE) and 1% DMF as cosolvents in the presence or absence of antibody. Assays were conducted using spectrofluorimetry (VTT1E3) by observing fluorescence enhancement (λ_{ex} = 337 nm, λ_{em} = 410 nm) or using chymotrypsin-coupled visible spectroscopy (VTT35C8) by observing the increase in absorbance due to the formation of *p*-nitroaniline (λ = 392 or 426 nm). [b] Prepared as stock solutions in 470 mM LiCl in TFE; cis:trans: **3**, 50:50; **4**, 61.39; **5**, 49:51; **6**, 28:72. Due to low substrate solubility, the K_m values had to be assessed over a narrow concentration range (0.5 K_m to 1.5 K_m) and were estimated to be near 100 μM in all cases. The P_1 valyl analogue of **5** was too insoluble in TFE/LiCl and that of **6** reacted too rapidly with chymotrypsin.

hydrophobic effects.[14] The rate enhancements produced here can be compared to the 46-fold increase for proline model compounds in going from water to toluene.[9a] In addition, given only 2 of 28 mAbs showed activity with unique substrate fidelities makes it likely that geometric influences induced by the twisted-dicarbonyl moiety are also necessary for antibody catalysis. This is further reflected in both the apparent preferential binding of the cis isomer and the less polar P_1−alanyl compound **3** being a better substrate than **4** that indicate factors other than simple hydrophobic interactions are involved. The function of the ketoamide may be coupled in a subtle way to dielectric effects and other catalytic phenomena such as transition-state stabilization and ground-state destabilization. Indeed, calculations demonstrate that these principles along with conformational constraints (autocatalysis) play a major role in FKBP catalysis.[15]

Although k_{cat}/K_m and k_{cat} for these antibodies are far less in relation to those of cyclophilin (10^5-fold), they are only 10^3 times slower than those for FKBP using optimum tetrapeptides and within an order of magnitude of the enzyme's rates with its poorer substrates. The FKBP activity is much more influenced by hydrophobic factors than is that of cyclophilin which may invoke minor alterations in its mode of catalysis.[13a,15b,16] That the antibody models can begin to approach FKBP catalysis implicates perhaps a more primitive isomerase activity for FKBP compared to that for cyclophilin.

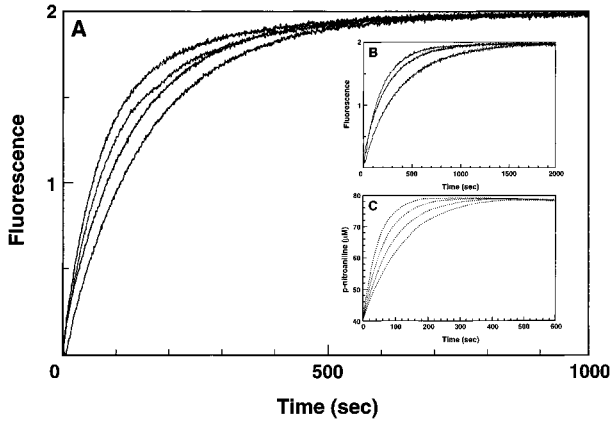


Figure 1. Representative progress curves for the antibody-catalyzed cis to trans isomerization of tripeptides. (A) (Top curve to bottom curve) 10, 5, 2.5, 0 μM mAb VTT1E3; 200 μM **3**. (B) (Top to bottom) 10, 5, 0 μM mAb VTT1E3; 100 μM **4**. (C) (Top and bottom) 10, 5, 2.5, 0 μM mAb VTT35C8; 200 μM **5**. Conditions are described in Table 1. All experiments were reproducible to within ±5%.

The investigation described herein supports the hypothesis of other workers that the α-ketoamide functional group in immunophilin ligands evolved to provide a contributing element in immunophilin rotamase activity. Additional hapten modifications can further address this issue and enable other features of catalysis to be examined. The catalytic antibodies derived from these new designs will then give more insight into the unique mechanisms of the enzymatic isomerases.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM 43858) and by TEKES, Technology Development Centre, The Finnish Centre for International Mobility and Exchange Programmes, Helsinki, Finland.

Supporting Information Available: The synthesis of the hapten, competitive inhibitor, and substrates and the description of kinetic assay procedures (26 pages). Ordering information is given on any current masthead page.

JA954206P

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