COMMUNICATION

The α-ketoamide group: a new motif for the elicitation of catalytic antibodies for acyl-transfer reactions

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Received (in Cambridge) 29th January 1999, Accepted 29th March 1999

An α-ketoamide hapten elicited a monoclonal antibody that catalyzed the hydrolyses of esters and carbamates *via* either a covalent intermediate or direct addition of water.

The most successful haptens for the induction of catalytic antibodies with hydrolytic activity have been transition-state analogues based on phosphonate structures.[1] According to hypothesis and design, the phosphonate approach programmed the direct addition of a water molecule to appropriate substrates. However, in some cases, antibodies with nucleophilic side chains used for covalent catalysis were serendipitously elicited by phosphonate haptens.[2] Recently, a rational approach to the design of haptens for eliciting covalent catalysis was developed and coined "reactive immunization".[3] The methodology enabled the development of catalytic antibodies for enantioselective ester hydrolyses with high catalytic proficiencies.[4]

The α-ketoamide-containing natural product cyclotheonamide A inactivated the serine protease α-thrombin through formation of a hemiketal adduct between the hydroxy group of the catalytic serine and the ketone of the inhibitor.[5] Protease and esterase inactivation *via* such a mechanism was also observed using various trifluoromethyl ketones.[6] Reactive immunization using an α-ketoamide should elicit antibodies capable of operating by covalent catalysis through a discrete acyl-antibody intermediate. However, an additional factor generally encountered in the α-ketoamide structure is a ~90° twist angle about the carbonyl bonds that might mimic characteristics of a tetrahedral transition state.[7] This feature could make contributions to catalysis of covalent intermediate formation or direct hydrolysis.[8]

We previously determined the X-ray structure of **1** that revealed the presence of the keto form in the solid state with a dihedral angle of 92.6° about the two carbonyls similar to the angle observed in other α-ketoamides.[7,9] Under physiological conditions (deutero-PBS, pH 7.4, 10% DMSO-d_6 for solubility), [^{13}C]-labeled hapten analog **2** was found to exist predominantly (>97%) in the keto form (ketone, δ 192.7 ppm; hydrate, δ 94.5 ppm). According to the precedent for enzymatic inactivation, hapten **3** should be suitable for eliciting antibody catalysts for acyl-transfer reactions *via* a mechanism operating through a covalent intermediate.

A panel of 22 mAbs (monoclonal antibodies) was derived from immunization of mice with hapten **3** coupled to KLH (keyhole limpet hemocyanin). Antibodies were screened† for their ability to catalyze the hydrolysis of esters **4** and **6**. Two antibodies were found to accelerate ester hydrolysis and one antibody, mAb 3H11, was studied in detail. BIAcore (Pharmacia) analysis using hapten **3** tethered with an amine-linker[9] was used to determine k_{on} and k_{off} that provided a value of K_d = 10 nM and indicated excellent recognition and affinity of the α-ketoamide structure. The position of the substrate carbonyl group and other chemical features were varied and this afforded other substrates **5**, **7** and **8**.

The acetate ester **6** in the presence of mAb 3H11 produced a kinetic profile having a burst of 4-isobutyramidophenol release followed by a slower steady-state phase (k_{cat}/k_{uncat} = 10). For mechanisms invoking an acyl intermediate with deacylation as the rate-determining step, addition of a good nucleophile should increase the rate of deacylation and therefore the k_{cat}.[10] Consistent with this, addition of hydroxylamine increased the steady-state rate of the phenol release three-fold. The results suggested that reactive immunization had positioned a nucleophile appropriate for acylation reactions in the combining site of the antibody.

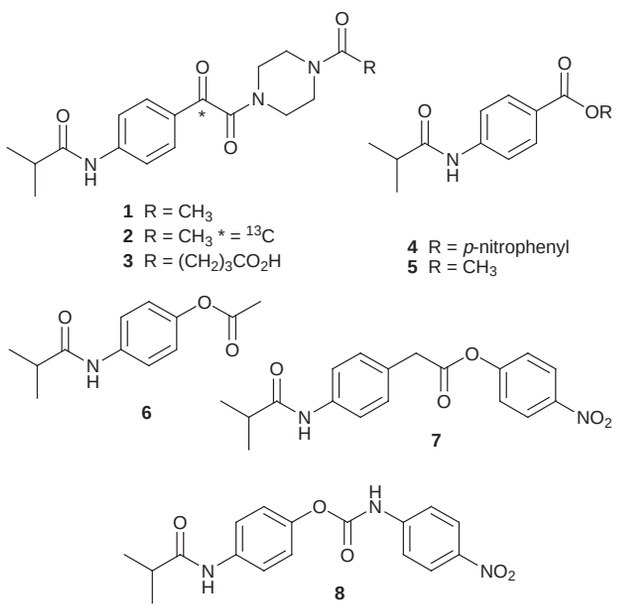
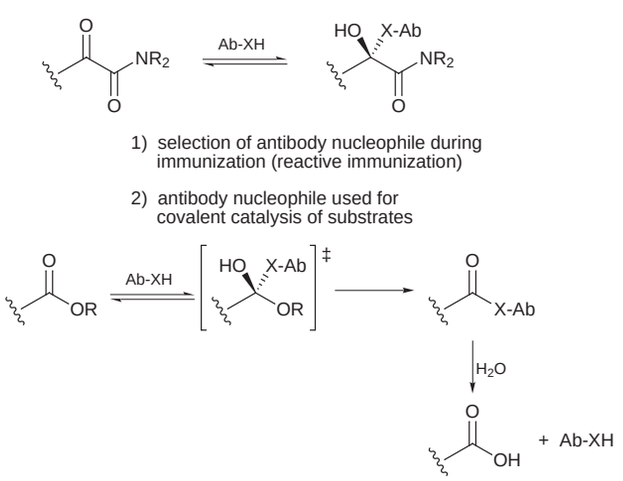


Fig. 1



Scheme 1

Initially, the reaction of mAb 3H11 with *p*-nitrophenyl ester **4** (5 μM mAb, 100 μM substrate) was observed to produce a burst of *p*-nitrophenol (v_{obs} = 1.0 μM min^{-1}) with a corresponding lag in acid production (v_{obs} = 0.02 μM min^{-1}). A complete Michaelis–Menten analysis was not feasible due to substrate solubility limitations, but the initial rates in the concentration range studied were linear with antibody concentration and the reaction was inhibited by hapten analog **1**. Again, the kinetic profile suggested the presence of an acyl-antibody in which breakdown of the intermediate was the rate-determining step. However, HPLC experiments indicated a discrepancy in the amount of *p*-nitrophenol and 4-isobutyramidobenzoic acid produced in the antibody-catalyzed reaction. The absence of one-half equivalent of acid suggested either covalent or tight noncovalent binding of the acyl portion to the antibody. Quenching the reaction under strongly denaturing conditions (6 M guanidinium hydrochloride) gave no increase in the amount of acid so that the data were in accord with covalent, irreversible acylation of the antibody.

The above "suicide" type of inhibition had been observed previously for esterolytic antibody catalysts.[11] Essentially, the ester **4** was a mechanism-based inactivator of mAb 3H11 where antibody acylation (k_{acyl}) was in competition with substrate hydrolysis (k_{cat}). A qualitative comparison of the rates of *p*-nitrophenol and acid production indicated that antibody acylation was the predominant pathway. A comparison of the estimated k_{acyl} and k_{cat} for **4** to the measured k_{uncat} (6.7×10^{-5} min^{-1}) provided a lower limit for the rate accelerations of 1490 and 30, respectively. The dual activity observed could result from alternative binding modes as determined by the two twisted atropisomers possible for hapten **3**, yielding different mechanistic pathways, or competition in a single binding mode between addition of an antibody nucleophile and of water.

Interestingly, the methyl ester **5** with the carbonyl and 4-isobutyramidophenyl group located identically to that of **4** was also a substrate for mAb 3H11 ($k_{cat} = 2.0 \times 10^{-4}$ min^{-1}, K_m = 280 μM, k_{cat}/k_{uncat} = 100). Notably, the antibody was not inactivated during reaction with **5**, so that now simple hydrolysis was the sole mechanistic pathway. Similarly, in the reaction with the *p*-nitrophenol ester **7**, no burst of *p*-nitrophenol was detected and equimolar amounts of acid and *p*-nitrophenol were produced. Hence, **7** did not irreversibly acylate the antibody as for **4**, although the possibility of an acyl intermediate could not be excluded. The varied and unusual reactivity of the closely related activated esters **4** and **7** demonstrated the subtle differences in kinetic behavior afforded in an antibody elicited by the α-ketoamide hapten.

The aryl carbamate **8** was a substrate for antibody mAb 3H11 (k_{cat} = 0.20 min^{-1}, K_m = 120 μM, k_{cat}/k_{uncat} = 480). The reaction yielded multiple turnovers, no indication of product inhibition and was competitively inhibited by **1** (K_i = 0.5 μM). No burst was observed and the rates of 4-isobutyramidophenol and 4-nitroaniline formation were identical by HPLC analysis which indicated production of the phenol was in the steady-state. However, both the log V_{max} and log V_{max}/K_m *versus* pH profiles showed an apparent pK_a = 7.5 where the deprotonated form of an amino acid residue was catalytically active. The energetically favored, uncatalyzed hydrolytic pathway for aryl carbamates is believed to proceed by an elimination (E1cB) mechanism forming an alcohol and isocyanate in which the latter then reacts with water and decarboxylates to form the amine and carbon dioxide.[12] The disfavored pathway is an addition–elimination ($B_{AC}2$) mechanism proceeding through a tetrahedral intermediate. Antibodies have been shown to catalyze the highly disfavored $B_{AC}2$ process.[13] While it remains to be determined, here the elimination mechanism might be favored since hapten design elements to dictate otherwise were not incorporated. Hence, the active-site nucleophile participating in acylation with esters **4** and **6** might act as a general base for carbamate hydrolysis.

In the light of the known structure and reactivity of α-ketoamides, the data supported a reactive immunization response to the hapten along with possible modes of transition-state stabilization. However, further studies will be required to unequivocally address this issue. Given the successful results, a new model is now available for obtaining antibody catalysts for ester and carbamate hydrolyses, and for the exploration of other acyl-transfer reactions.

Acknowledgements

Mrs Marie-Rose Benitez for antibody production, Mrs Anja Salakari for technical assistance, and Shenlan Mao for BIAcore analysis. J. Y.-K. thanks TEKES (Technology Development Centre, Finland). The Skaggs Institute for Chemical Biology, the National Institute of Health (GM-43858) and The Scripps Research Institute.

Notes and references

† Determined at 22 °C in 50 mM phosphate–100 mM NaCl, pH 8.0, with 5% DMSO as cosolvent for **5** and **6**, and 4% DMF, 1% dioxane, 1% Tween-80 for **4**, **7** and **8**.

‡ BIAcore analysis is a system for real time bimolecular interaction analysis (BIA) using surface plasmon resonance technology.

1 P. G. Schultz and R. A. Lerner, *Science*, 1995, **269**, 1835; B. J. Lavey and K. D. Janda, *Antibody Expression and Engineering*, ACS Symposium Series 604, 1995, ch. 10.
2 J. Guo, W. Huang and T. S. Scanlan, *J. Am. Chem. Soc.*, 1994, **116**, 6062; M. T. Martin, A. D. Napper, P. G. Schultz and A. R. Rees, *Biochemistry*, 1991, **30**, 9757; P. Wirsching, J. A. Ashley, S. J. Benkovic, K. D. Janda and R. A. Lerner, *Science*, 1991, **252**, 680.
3 P. Wirsching, J. A. Ashley, C.-H. L. Lo, K. D. Janda and R. A. Lerner, *Science*, 1995, **270**, 1775.
4 C.-H. L. Lo, P. Wentworth, Jr., K. W. Jung, J. Yoon, J. A. Ashley and K. D. Janda, *J. Am. Chem. Soc.*, 1997, **119**, 10251.
5 S. D. Lewis, B. J. Lucas, S. F. Brady, J. T. Sisko, K. J. Cutrona, P. E. J. Sanderson, R. M. Freidinger, S.-S. Mao, S. J. Gardell and J. A. Shafer, *J. Biol. Chem.*, 1998, **273**, 4843; B. E. Maryanoff, X. Qiu, K. P. Padmanabhan, A. Tulinsky, H. R. Almond, Jr., P. Andrade-Gordon, M. N. Greco, J. A. Kauffman, K. C. Nicolaou, A. Liu, P. H. Brungs and N. Fusetani, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 8048.
6 K. Brady, A. Wei, D. Ringe and R. H. Abeles, *Biochemistry*, 1990, **29**, 7600; K. N. Allen and R. H. Abeles, *Biochemistry*, 1989, **28**, 8466; B. Imperiali and R. H. Abeles, *Biochemistry*, 1986, **25**, 3760.
7 R. D. Bach, I. Mintcheva, W. J. Kronenberg and H. B. Schlegel, *J. Org. Chem.*, 1993, **58**, 6135.
8 J. T. Yli-Kauhaluoma and K. D. Janda, *Bioorg. Med. Chem.*, 1994, **2**, 521.
9 M. J. Taylor, T. Z. Hoffman, J. T. Yli-Kauhaluoma, R. A. Lerner and K. D. Janda, *J. Am. Chem. Soc.*, 1998, **120**, 12783.
10 A. Fersht, *Enzyme Structure and Mechanism*, W. H. Freeman, NY, 1985, 2nd edn.
11 T. S. Angeles and M. T. Martin, *Biochem. Biophys. Res. Commun.*, 1993, **197**, 696.
12 A. F. Hegarty and L. N. Frost, *J. Chem. Soc., Perkin Trans. 2*, 1973, 1719.
13 P. Wentworth, Jr., A. Datta, S. Smith, A. Marshall, L. J. Partridge and G. M. Blackburn, *J. Am. Chem. Soc.*, 1997, **119**, 2315.

Communication 9/02475A