Catalytic Properties of the Antibody H11

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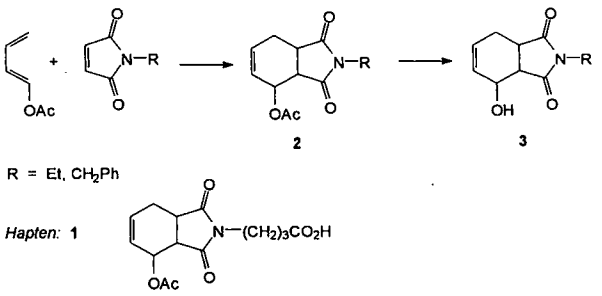
Abstract. The catalytic activity of the antibody H11 is shown to reside chiefly in its ability to hydrolyze 1-acetoxybutadiene to crotonaldehyde and to promote the cycloaddition of the intermediate enol with *N*-alkylmaleimides. This conclusion is based upon the demonstration that the enol tautomerizes too rapidly in solution to be a competent intermediate and that under the reaction conditions for H11, no cycloaddition occurs with crotonaldehyde and *N*-ethylmaleimide. As a first step towards a structural understanding of the chemistry of H11, chemical modification experiments have shown that reactions of acidic amino acids, tyrosine, lysine, and histidine, but not arginine, inhibit the reactions mediated by H11.

INTRODUCTION

One of the first reactions shown to be catalyzed by antibodies was the Diels–Alder reaction,[1-3] principally because of the well-defined product-like transition state from which haptens could be designed and the fact that at that time, no enzymes catalyzing the Diels–Alder reaction had been described.[4] With the potential of organic synthesis in mind, we selected a Diels–Alder cycloaddition that led to a polyfunctional bicyclic system for study (Scheme 1). In common with other examples,[1,2] reactions of *N*-alkylmaleimides were chosen; the intention was that product inhibition might be avoided by suitable choice of the *N*-alkyl group. Antibodies were raised to the hapten **1** (Scheme 1) following standard protocols and were screened for chemical reactivity by following the loss of *N*-alkyl maleimide by HPLC. A number of antibodies that responded to this screen were identified, and one of them, H11, was selected for more detailed study.[5]

The original kinetic evaluation of H11 was carried out using a development of the screening assay following the rate of loss of *N*-ethylmaleimide. At that time, we were unable to assay the rate of formation of the acetoxy adduct **2** effectively although its presence could be demonstrated. When preparative-scale reactions were carried out to isolate and characterize products spectroscopically, we identified, surprisingly, the hydroxy adduct **3** together with the acetoxy adduct **2**. The hydroxy adduct was found to be produced when the acetoxy adduct was incubated with H11 or with its Fab fragment (prepared using immobilized papain digestion), a reaction that was inhibited measurably by added *N*-alkylmaleimide.[5] On the basis of this information, we proposed that H11 catalyzed two reactions, namely hydrolysis of the acetate and cycloaddition as planned. The reliance on a reactant-based assay was a weakness in this proposal and it was also unexpected that significant product inhibition was not observed.

The conclusions were summarily criticized and the behavior observed was attributed to contaminating proteins.[6] While it was entirely reasonable to point out the limitations in the conclusions that could be drawn from the assay used, the comments about antibody purity were speculative. Indeed, all of the reactions described



Scheme 1

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for native H11 were found to be catalyzed by its Fab fragment. It was further suggested, contrary to the accumulated body of synthetic chemical knowledge, that the hydroxy adduct **3** could be formed by cycloaddition of the *N*-alkylmaleimide with the enol of crotonaldehyde, the hydrolysis product of the starting material, acetoxybutadiene. To the best of our knowledge, such a reaction has never been described. The normal reactions of crotonaldehyde in cycloadditions are either as the dienophile, in which formyl-substituted compounds are formed, or as a heterodiene, in which tetrahydropyrans are formed.[7] We describe below experiments that clarify the chemical reactions catalyzed by H11.

EXPERIMENTAL

(Z)-*1-Trimethylsilyloxybuta-1,3-diene* **4**

4 was prepared as previously described.[8,9]

Reactions of (Z)-trimethylsilyloxybuta-1,3-diene **4**

Stability of the enol **5**: *NMR experiments.* The NMR spectrum (250 MHz) of a solution of **4** (10.6 mg) in CD_3CN (0.5 mL) was obtained; characteristic resonances of **4** were observed at d 6.18, 5.24, 5.08, and 4.91. Addition of DCl (10 μL, 20% solution) led to complete, virtually instantaneous hydrolysis of **4** as shown by the disappearance of the resonances of **4** and the appearance of resonances characteristic of crotonaldehyde. Hydrolysis was also observed by the addition of 0.2% DCl (1 μL). In contrast, addition of phosphate buffered saline (PBS) buffer in D_2O (pH 7.4, 0.2 μL) did not lead to hydrolysis over a period of many minutes. The enol **5** (O-deuteriated) was generated by adding 0.02% DCl (0.1 μL) to a solution of **4** in CD_3CN (concentration as above) at room temperature and was stable as shown by NMR for more than 30 min. In contrast, a further sample prepared in this way was treated with PBS in D_2O (1.5 mL); under these conditions, **5** was not observed.

Stability of the enol **5**: *effect of solvent composition.* The hydrolysis of **4** to **5** and its subsequent tautomerization to crotonaldehyde was followed by UV spectrophotometry measuring the absorbance at 237 nm as described by Capon and Guo.[8]

(a) In CH_3CN alone. Solutions of **4** (30 mg) in CH_3CN (1.5 mL) were treated with HCl (4.6 μL, 1.026 M to give a concentration of 3.16 mM) and the decrease in absorbance followed over 99 min. A steady decrease over the first 3–4 min was observed, after which time the absorbance became constant.

(b) In H_2O/CH_3CN. Solutions of **4** (85 mg) in H_2O/CH_3CN (1:9 v/v) were prepared and observed by UV. Under these conditions, hydrolysis was slow, a constant absorbance being reached only after about 80 min.

(c) In PBS buffer/CH_3CN. A series of samples of **4** (60 mg) dissolved in PBS buffer (pH 7.4) and CH_3CN in the following compositions (v/v) 10:90, 20:80, 25:75, 30:70, 40:60; 50:50; 60:40, 70:30, and 75:25 to give a total volume of 1.5 mL. The initial absorbance at 237 nm was 2.49. For compositions up to and including 30:70, the absorbance remained essentially constant over 99 min. For 40:60 and 50:50 compositions, a slow decrease in absorbance was measured ($t_{1/2}$ ~ 15 min). For the remaining compositions, the absorbance at 237 nm had decreased essentially to base level before measurements could be begun.

Determination of kinetic parameters for hydrolysis of acetoxybutadiene by H11

(Experiments carried out by Sandeep Naidu.) Reactions were carried out in aqueous PBS (pH 7.4)/CH_3CN (3:1 v/v) at 37 °C, observing the loss of acetoxybutadiene at 250 nm. [Acetoxybutadiene] was in the range 4.76×10^{-5} M to 2.38×10^{-4} M and [H11] was 9.25×10^{-8} M.

HPLC assays of products of reactions of acetoxybutadiene were run on octadecylsilyl reverse phase columns, flow rate 1 mL min^{-1}, eluting with $CH_3CN:H_2O$ 1:1 v/v, observing at 210 nm. The low wavelength detection was essential to observe all products. Under these conditions, typical retention times (min) at ambient temperature were: *N*-ethylmaleimide 4.7, acetoxybutadiene 9.4, **2** 5.2, **3** 4.0, crotonaldehyde 4.3.

Chemical modification of H11. Reactions were carried out at 30 °C under the conditions stated below:

Histidine: [diethylpyrocarbonate] 0.353 mM, [H11] 3.53 mM, 0.1M PBS, pH 7.5

Arginine: [phenylglyoxal] 1 mM, [H11] 0.01 mM, 0.1 M PBS, pH 8.1

Tyrosine: [tetranitromethane] 1 mM, [H11] 0.01 mM, 0.1 M PBS, pH 7.5

Lysine: [trinitrobenzenesulphonate] 1 mM, [H11] 0.01 mM, 0.1 M PBS, pH 7.5

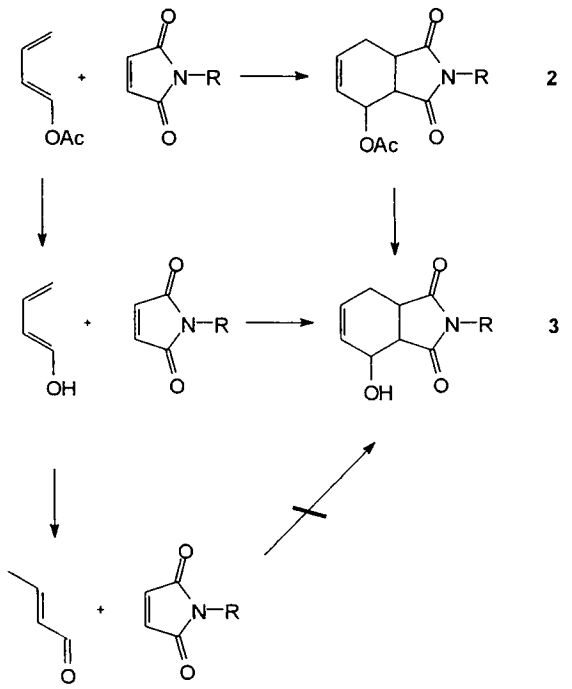
Aspartic and glutamic acids: [*N*-3-(dimethylamino)propyl-*N*-ethylcarbodiimide] 0.057 M, [H11] 5.71 mM, [NH_4Cl] 0.057 mM, 0.1M PBS, pH 4.7.

After the appropriate incubation time, low molecular weight compounds were removed by gel filtration (Sephadex G-25), centrifuged, concentrated, and the supernatant solutions assayed for catalytic activity. These reactions were carried out using [H11] 0.01 mM, [*N*-ethylmaleimide] 1 mM, and [acetoxybutadiene] 1.5 mM, assayed by HPLC as described above.

REACTIONS CATALYZED BY H11

Despite the body of precedent, we first demonstrated that crotonaldehyde failed to undergo cycloaddition with *N*-alkylmaleimides in a variety of organic solvents at reflux and under the conditions of reaction with H11, namely acetonitrile/PBS pH 7.4 (1:3 v/v) in the presence or absence of H11. An efficient HPLC assay was then devised in which both starting materials and products could be determined from a single run; the crucial modification was to detect products at low wavelength (210 nm). Using this improved experimental methodology we were able to show that (Scheme 2):

1. H11 catalyzes the hydrolysis of acetoxybutadiene to crotonaldehyde;



Scheme 2

2. The formation of the acetoxy adduct **2** is scarcely, if at all, catalyzed by H11;
3. However, the formation of the hydroxy adduct **3** is strongly catalyzed by H11.

We had already shown that the rate of hydrolysis of **2** to **3** by H11 is slow compared with the rate of cycloaddition.[5] Traces of other products were identified including the half ethylamide of maleic acid which is formed slowly in a background reaction. A typical reaction profile for the cycloadducts is shown in Fig. 1, which illustrates the reaction mediated by H11 in comparison with background (PBS buffer/acetonitrile), and with H11 modified by *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC)–ammonium chloride as a representative chemical modification (see below). The similarity between the background reaction and that with the chemically modified H11 is notable. The reaction profiles represented by Scheme 2 and Fig. 1 are also consistent with evidence for inhibition of H11-catalyzed hydrolysis of **2** by *N*-benzylmaleimide (IC_{50} ~ 30 mM)[5] and of H-11 catalyzed hydrolysis of acetoxybutadiene by **2** (IC_{50} ~ 2mM) but not by **3**.

Two possible origins for the hydroxy adduct **3** need to be considered. Either crotonaldehyde enol, generated by H11-catalyzed hydrolysis of acetoxybutadiene, is released into solution and is trapped by *N*-alkylmaleimide or the enol remains bound on the antibody surface and reacts with *N*-alkylmaleimide. Extensive work by Capon and Guo has shown that it is possible to prepare enols of crotonaldehyde in aprotic organic solvents such as acetone and acetonitrile, to characterize them spectroscopically, and to measure the rates of tautomerization to the keto form.[8] They have shown that under aqueous basic conditions, the rate constant for tautomerization exceeds 10^5 s^{-1}. It would therefore not be expected that a free enol would survive long enough to afford cycloaddion products. Nevertheless we have tested this possibility in two ways. H11 was incubated with acetoxybutadiene in the presence of electrophilic carbonyl compounds as alternative enol traps (ethyl pyruvate and trifluoroacetophenone) and cycloaddition/Michael acceptors (diethyl maleate, 5-dihydrofuran-2-one, and 1,4-benzoquinone); in no case was a new product detected. Secondly, the stability of a crotonaldehyde

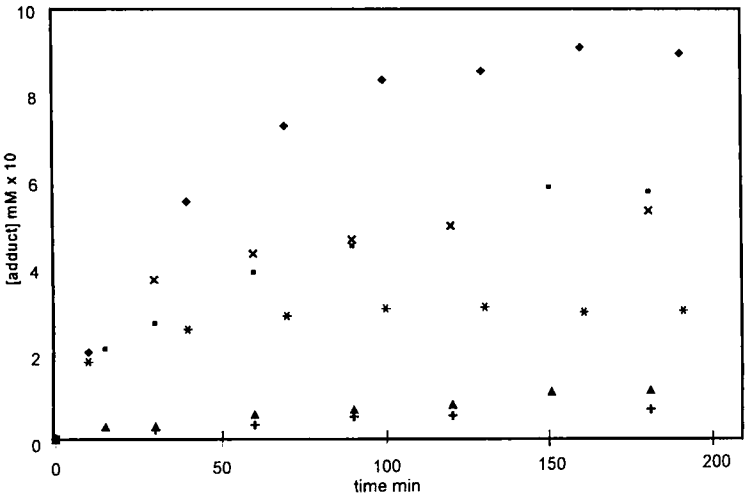
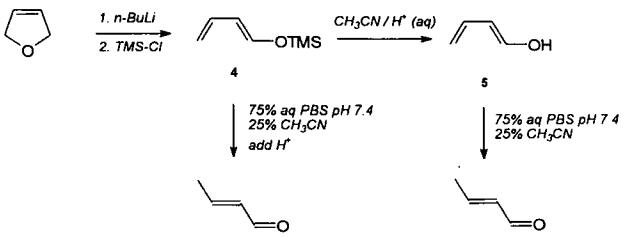


Fig. 1. Cycloadducts formed in presence and absence of H11 and with EDC–NH_4Cl-modified H11. ■ **2** background; ▲ **3** background; * **2** H11; ◆ **3** H11; x **2** EDC-modified; + **3** EDC-modified.

enol under our reaction conditions for H11 was investigated. Following Capon and Guo, the trimethylsilylether **4** was prepared from dihydrofuran[9] and converted into the corresponding enol by the addition of trace quantities of acid. The enol itself could be observed by NMR and UV spectroscopy (λ_{max} 237 nm). We found that in solvent mixtures in which the major component was acetonitrile, the half-life of the enol was approximately 15 min, but under the conditions of our reactions (75% PBS), the enol had completely tautomerized within 1–2 min before the spectroscopic measurement could be begun. Both these results are consistent with cycloaddition of the enol bound to the surface of the antibody and not with cycloaddition occurring in solution.

The current understanding of the reaction sequence catalyzed by H11 is shown in Scheme 2. Because of the uncertainties surrounding this scheme, we have not yet made quantitative measurements of all of the individual rates of reactions. Recently, we have established catalytic constants for the hydrolysis of acetoxybutadiene at 30 °C: K_M = 0.027 mM, k_{cat}/k_{solv} =7000. The previous results,[5] in which the total loss of maleimide was measured, showed that the overall processing of *N*-ethylmalimide by H11 was associated with a K_M = 8.3 mM. It appears, therefore, that H11 is indeed a dual catalyst and catalyzes the hydrolysis of acetoxybutadiene together with a reaction not previously described, the cycloaddition of a dienol and a dienophile. This scheme also helps to account for the lack of product inhibition, since the major product is the hydroxy adduct **3**, and not the acetoxy adduct **2** which is a substructure



Scheme 3

of the hapten **1**. The pH dependence previously reported[5] can also be understood as associated with acetoxybutadiene hydrolysis. However, we are still struggling to find a reliable method for determining the chirality, if any, of the products. Many chromatographic and derivatization procedures have been investigated without success.

To move towards a structural understanding of the reactions catalyzed by H11, experiments with chemical modifying reagents were carried out. Reactions targetted at histidine (diethylpyrocarbonate),[10] lysine (trinitrobenzene sulphonate),[11] tyrosine (tetranitromethane),[12] glutamate/aspartate (ammonium chloride/carbodiimide),[10] and arginine (phenylglyoxal)[13] were carried out. All reagents except phenylglyoxal caused a high degree of inhibition of all of the reactions catalyzed by H11. The reaction profile of the modified H11 samples was unlike that of unmodified H11 and typical of the background reaction (Figs. 1 and 2). For all modifications except carbodiimide, evidence for time-dependent inhibition was found. An example is shown in Fig. 3 for the reaction of histidine with diethylpyro-

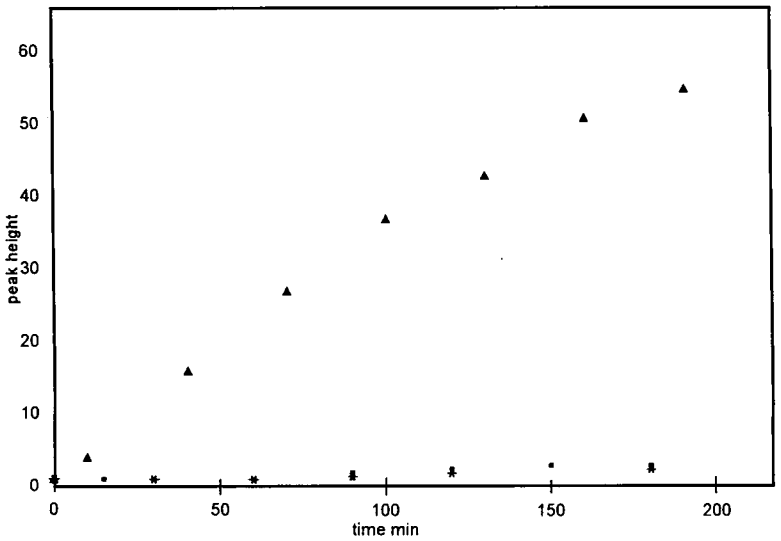


Fig. 2. Crotonaldehyde formed by H11 (▲), EDC-modified H11 (*), and in background (■).

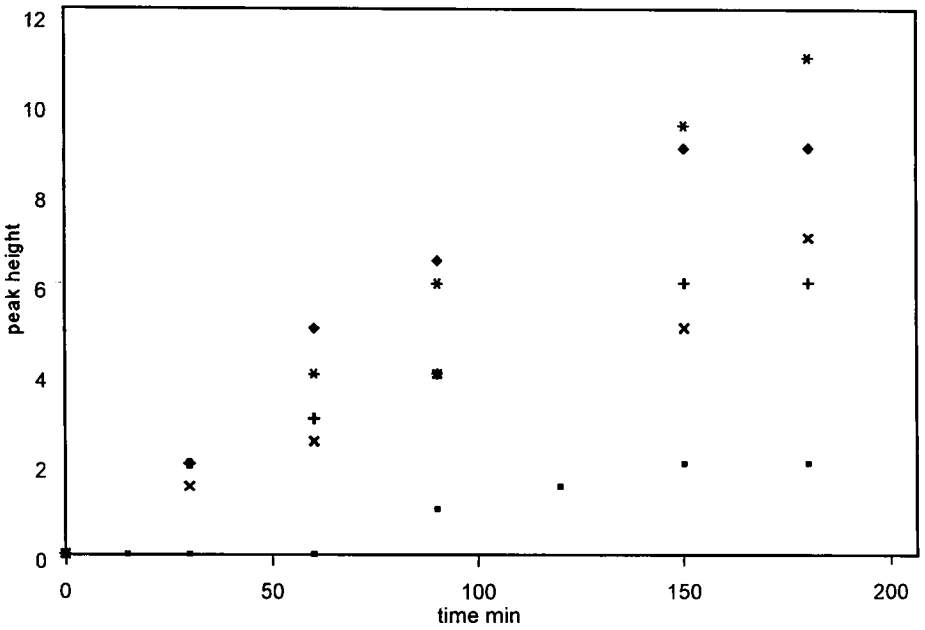


Fig. 3. Inhibition of hydrolysis of acetoxy-butadiene catalyzed by H11 (3.53 mM) caused by modifying histidine residues with diethyl-pyrocarbonate (0.353 mM). ■ background; * 30 min preincubation with diethylpyrocarbonate; ◆ 60 min; x 120 min; +180 min.

carbonate. The most effective reagent was ammonium chloride/carbodiimide which virtually abolished the production of crotonaldehyde and the hydroxy adduct **3** within 30 min, the shortest preincubation time used in these experiments. Further experiments will be carried out to determine whether any of these inhibition reactions are closely associated with the hapten binding site. It is probable, however, that this binding site has a high degree of functionality in contrast to that of the catalytic antibody C3 that we previously described.[14]

The catalytic properties of H11 can therefore be interpreted in terms of a major reaction pathway involving initial hydrolysis of acetoxybutadiene followed by trapping of the enol product by bound maleimide which takes place in competition with release of the enol and tautomerism to crotonaldehyde. Enol intermediates and products have been described for enzyme-catalyzed reactions in carbohydrate and ketoacid metabolism.[15] There is some evidence that the tautomerism of enolpyruvate is enzyme catalyzed.[16] With regard to H11, there is no evidence concerning the precise timing of tautomerization except that it has proved impossible so far to trap the enol with any reagent other than a maleimide. H11 thus shows two especially interesting properties, namely, the catalysis of two reactions of differing types and the promotion of a reaction that has not otherwise been shown to occur.

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REFERENCES AND NOTES

(1) Hilvert, D.; Hill, K.W.; Nared, K.D.; Auditor, M.-T.M. *J. Am. Chem. Soc.* **1989**, *111*, 9261.
(2) Braisted, A.C.; Schultz, P.G. *J. Am. Chem. Soc.* **1990**, *112*, 7430.
(3) Gouverneur, V.E.; Houk, K.N.; Pascual-Teresa, B.; Beno, B.; Janda, K.D.; Lerner, R.A. *Science* **1993**, *262*, 204.
(4) Oikawa, H.; Katayama, K.; Suzuki, Y.; Ichihara, A. *J. Chem. Soc., Chem. Commun.* **1995**, 1321.
(5) Suckling, C.J.; Tedford, M.C.; Bence, L.M.; Irvine, J.I.; Stimson, W.H. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1925.
(6) Hilvert, D. *Acc. Chem. Res.* **1993**, *26*, 552.
(7) Fernandez, J.E.; Solomons, E.W.G. *Chem. Rev.* **1962**, *62*, 485.
(8) Capon, B.; Guo, B. *J. Am. Chem. Soc.* **1988**, *111*, 5144.
(9) Bates, R.B.; Kroposki, L.M.; Potter, D.E. *J. Org. Chem.* **1972**, *37*, 560. Oakes, F.T.; Yang, F.A.; Sebastian, J.F. *J. Org. Chem.* **1982**, *47*, 3094. Rautenstrauch, V. *Helv. Chim. Acta* **1972**, *55*, 594.
(10) Gudmundsson, B.-M.E.; Young, N.M.; Oomen, R.P. *J. Immunol. Methods* **1993**, *158*, 315.
(11) Fields, R. *Methods Enzymol.* **1972**, *25*, 464.
(12) Riordan, J.F.; Vallee, B.L. *Methods Enzymol.* **1972**, *25*, 515.
(13) Takahashi, K. *J. Biol. Chem.*, **1968**, *2443*, 6171.
(14) Khalaf, A.I.; Proctor, G.R.; Suckling, C.J.; Bence, L.H.; Irvine, J.I.; Stimson, W.H. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1475.
(15) Rose, I.A. *Methods Enzymol.* **1982**, *87*, 84.
(16) Burgner, J.W.; Ray, W. *Biochemistry* **1874**, *13*, 4229.