Detection of a Catalytic Antibody Species Acylated at the Active Site by Electrospray Mass Spectrometry†

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ABSTRACT: The chemical interactions between a catalytic antibody Fv fragment and ester substrates were examined using pneumatically assisted electrospray (ion spray) mass spectrometry. Upon addition of the *p*-nitrophenyl ester substrate to the antibody fragment, an antibody fragment species that represents approximately 8% of the total Fv concentration is clearly observed in the electrospray spectrum. The observed increase in molecular weight of the Fv fragment corresponds to the mass of the acyl group of the substrate. Formation of the acyl−Fv species is blocked by preincubation of the antibody fragment with hapten inhibitor, suggesting that the acyl linkage involves a residue in the active site of the antibody. The acyl−Fv species is not observed when the corresponding *p*-chlorophenyl ester substrate is used, indicating that the level of this species is dependent on the leaving group of the substrate. The acylated species is not observed for a site-directed mutant lacking catalytic activity, His L91 Gln. The present results are consistent with modeling studies of the structure of the Fv fragment and provide strong confirmatory evidence for the multistep kinetic mechanism previously proposed for this antibody.

NPN43C9 is a catalytic antibody which catalyzes the hydrolysis of an aromatic anilide and a series of related phenyl esters (Janda *et al.*, 1988). This antibody was induced by a phosphonamidate hapten (**1**) which resembles the tetrahedral transition state for ester (**2a, 2b**) or anilide (**2c**) hydrolysis.

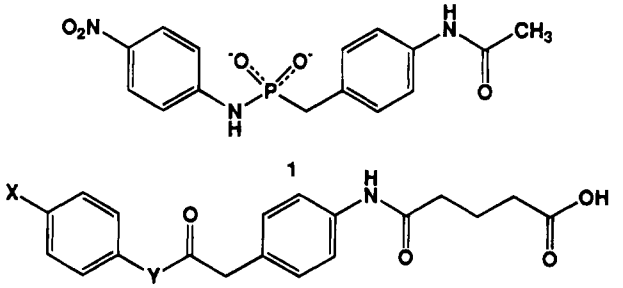
Recent computer modeling and mechanistic studies of NPN43C9 have provided considerable insight into its catalytic mechanism (Benkovic *et al.*, 1990; Janda *et al.*, 1991; Gibbs *et al.*, 1992; Roberts *et al.*, 1994). In the proposed kinetic scheme, NPN43C9 catalyzes hydrolysis through a multistep mechanism (Scheme 1) involving an

Scheme 1

$$\mathrm{Ab + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{AbS} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AbIP} \underset{k_{-3}}{\overset{k_3[OH^-]}{\rightleftharpoons}} \mathrm{AbPP'} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}}$$

$$\mathrm{AbP + P'} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{Ab + P}$$

attack on the carbonyl carbon atom of the ester or anilide substrate by a nucleophile believed to be the imidazole group of the histidine residue of the light chain of the antibody (His L91). This attack results in the formation of an acyl−antibody intermediate, which is decomposed by hydroxide ion to form the products, which dissociate in a sequential



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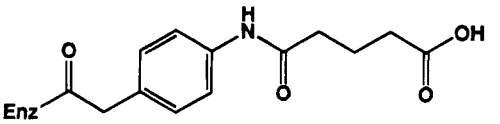
2a-c

a: X = NO_2, Y = O
b: X = Cl, Y = O
c: X = NO_2, Y = NH



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manner to regenerate the free, catalytically active form of the antibody. Although available kinetic data are consistent with the formation of the acyl intermediate, attempts to detect the intermediate spectrophotometrically have been unsuccessful (Benkovic *et al.*, 1990). Kinetic analysis suggested that such a species would only accumulate to a maximum extent of 5% of total antibody concentration at neutral pH.

Electrospray mass spectrometry is a highly sensitive technique for detecting covalent and tight-binding ($K_D \leq 100$ nM) protein complexes (Burlingame *et al.*, 1994; Siuzdak *et al.*, 1994). This technique is particularly well suited for examining covalent reaction intermediates of nucleophilic

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catalysis since it can be performed at acidic pH where hydroxide-mediated reactions are minimized, and it has been successfully employed to detect covalent reaction intermediates for several enzymes (Alpin *et al.*, 1990, 1992; Ashton *et al.*, 1991; Shneier *et al.*, 1991). Although this technique cannot be used for the intact catalytic antibody due to the antibody's high molecular mass (~150 kDa), it is ideally suited for examining smaller fragments such as the single-chain Fv fragment (scFv; Siuzdak *et al.*, 1994). We have utilized electrospray mass spectrometry to examine the chemical interactions of recombinant single-chain NPN43C9 Fv fragment with phenyl ester substrates. As a result, we have directly identified a novel species with the substrate acyl group covalently attached to the antibody fragment. This provides the first direct evidence for the acyl–antibody intermediate previously proposed on the basis of kinetic and mutagenesis data.

MATERIALS AND METHODS

Phosphonamidate hapten (**1**) and phenyl ester substrates (**2a**, **2b**) were synthesized as previously described (Gibbs *et al.*, 1992).

The gene encoding the NPN43C9 single-chain Fv fragment (Gibbs *et al.*, 1991) was placed under T7 phage transcriptional control by ligating the *SpeI*/*Bam*HI fragment of pJS118 (Stewart *et al.*, 1994) into the *XbaI*/*Bam*HI fragment of the pET22-b T7 expression vector (Novagen, Madison, WI) to create the expression plasmid pJK87.

For each protein preparation, pJK87 was transformed into BL21(DE3) cells containing pUBS520, a second plasmid encoding the AGA/G codon tRNA gene (Brinkmann *et al.*, 1989). The transformants were used to inoculate 1 L of rich induction medium (Krebs & Fierke, 1993) containing 100 μg/mL ampicillin and 25 μg/mL kanamycin sulfate, and the resulting culture was grown at 37 °C. When the culture A_{600} reached 1.0, IPTG was added to 1.2 mM and the culture was shaken at 25 °C for 16–20 h.

Cells were removed from the medium by centrifugation at 10000*g* for 15 min at 4 °C, and the medium supernatant was filtered through a 0.45-μm filter. After the addition of 1/20 vol of 1 M Tris·Cl, pH 8.0, protein was precipitated from the medium by adding 600 g/L ammonium sulfate and stirring for 60 min at 4 °C. The precipitate was collected by centrifugation at 10000*g* for 10 min, and the protein pellet was dissolved in a minimal volume (50–100 mL) of cold 20 mM MOPS and 0.4 mM $Ca(OAc)_2$, pH 6.2. This solution was dialyzed four times against 2 L of 20 mM MOPS and 0.4 mM $Ca(OAc)_2$, pH 6.2, at 4 °C and subjected to cation-exchange chromatography using a polyCATA column (Nest Group) as previously described (Gibbs *et al.*, 1991). Fractions containing the single-chain Fv fragment were collected, dialyzed against 2 L of cold 25 mM Tris·Cl and 50 mM NaCl, pH 8.0, and passed over a 3-mL DEAE-Sephacel column. To remove any remaining low molecular weight impurities, the sample was concentrated to 2.5 mL using ultrafiltration and passed over a desalting PD-10 gel filtration column (equilibrated with the same buffer). This procedure routinely produces 15–20 mg of homogeneously pure scFv as gauged by SDS–PAGE. The purified scFv was dialyzed twice against 2 L of cold, distilled, deionized H_2O and acidified to pH 5.9 with 0.01 M HCl immediately prior to electrospray analysis. Protein concentrations were determined by the absorbance at 280 nm using an extinction coefficient of 48 000 M^{-1} cm^{-1} (Gibbs *et al.*, 1991).

To study scFv–substrate interactions, 2.5 μL of 20 mM *p*-nitrophenyl or *p*-chlorophenyl ester (**2a, 2b**) in dimethylformamide was added to 97.5 μL of 10–30 μM scFv sample, and the mixture was rapidly injected (within 10–15 s of mixing) at a rate of 4 μL/min into a Perkin-Elmer SCIEX API III mass analyzer with pneumatically assisted electrospray ionization. The mass spectrum of the mixture was analyzed using declustering voltages in the range 75–250 V. For hapten-inhibited samples, a stoichiometric amount of phosphonamidate hapten (**1**) was added to the enzyme sample just prior to the addition of substrate. Due to the buffering effect of the protein, the pH of the reaction did not change significantly during the experiment (≤0.1 pH unit).

RESULTS

Under mildly acidic conditions (pH ≤ 6) and at very low ionic strength, recombinant NPN43C9 single-chain Fv fragment ($C_{1162}H_{1814}N_{316}O_{368}S_{10}$) provides a well-resolved electrospray mass spectrum (Siuzdak *et al.*, 1994). The experimentally observed mass, 26 419 ± 2.6 Da, is in good agreement with the calculated mass of 26 419.9 Da. The single-chain Fv readily binds hapten (Siuzdak *et al.*, 1994) and is catalytically active under these conditions [k_{cat} for the *p*-nitrophenyl ester at pH 5.9: 0.056 s^{-1} (observed under electrospray conditions) or 0.038 s^{-1} (observed in ATC buffer, 25 °C; Benkovic *et al.*,1990)]. Figure 1a shows the mass spectrum (*m/z* 2190–2240) of a 30 μM scFv sample at a declustering potential of 175 V. This spectral region contains an ion peak (*m/z* 2202) corresponding to the 12+ charge state. Other charge states are seen in the complete spectrum, but with lower intensity (Siuzdak *et al.*, 1994). The additional minor peaks observed near *m/z* 2204–2208 probably arise from sodium and/or calcium adducts. In order to maximize signal intensity for the detection of low concentrations of short-lived intermediates, subsequent spectra were acquired only in the region surrounding the 12+ mass peak.

Addition of 500 μM *p*-nitrophenyl ester (**2a,** M_r = 385 Da) to the scFv sample causes the appearance of an additional peak at *m/z* 2223 (Figure 1b). This peak is consistent with an adduct mass of 26 666 Da and represents 8% of the combined integrated scFv peak intensities after a brief (10–15 s) incubation on ice and immediate electrospray analysis at ambient temperature (20–25 °C). The observed mass increase of 247 Da is identical (within error) to the mass of the acyl functionality (**3**) of the substrate. The 26 666-Da species does not reflect the scFv–acid product complex since the *m/z* 2223 peak is not observed when 500 μM acid product is added to the scFv (Figure 1c). These spectra were acquired with declustering potentials of 175–250 V. Under these conditions, even high-affinity (≤1 nM) noncovalent complexes, such as the hapten–antibody complex, are not observed (Siuzdak *et al.*, 1994). The *m/z* 2223 peak is not observed if the scFv is preincubated with 30 μM hapten inhibitor prior to the addition of the substrate (Figure 1d). The covalent complex is not observed for the *p*-chlorophenyl ester substrate (**2b**) under a wide variety of reaction condi-

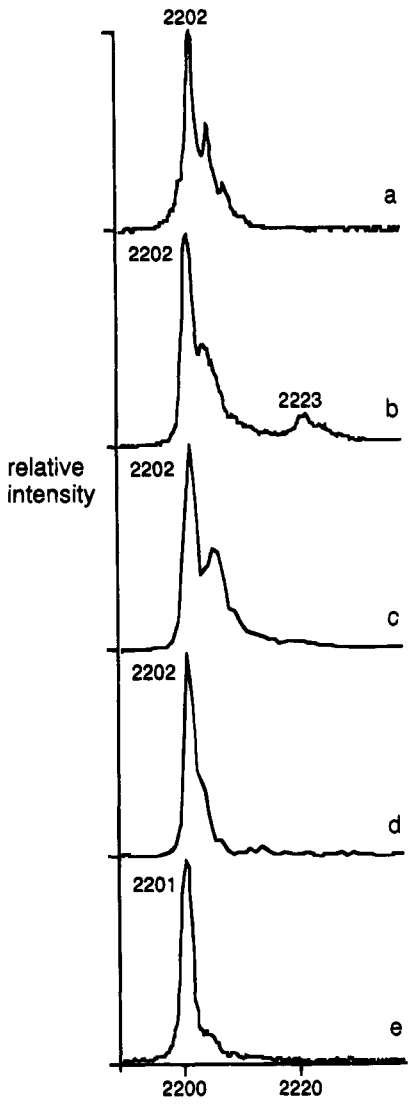

FIGURE 1: Electrospray spectra containing the 12+ charge peak of the single-chain Fv. Samples were prepared as described in Materials and Methods, and all spectra were acquired at an orifice potential of 175 V. (a) scFv only: (b) scFv in the presence of *p*-nitrophenyl ester substrate; (c) scFv with acid product; (d) scFv–hapten complex in the presence of *p*-nitrophenyl ester substrate; (e) scFv His L91 Gln variant in the presence of *p*-nitrophenyl ester substrate. Neither smoothing nor background subtraction has been used for these electrospray data.

tions (data not shown), indicating that the level of the acyl–scFv is highly dependent on the reactive nature of the leaving group. Finally, replacement of His L91, the putative nucleophile, with Gln [which eliminates catalytic activity but not hapten binding activity (Stewart *et al.*, 1994)] completely abolishes the minor peak at *m/z* 2233 (Figure 1e).

To test the consistency of our findings with previous kinetic studies of NPN43C9, the steady-state concentration of the acyl–catalytic antibody species was calculated with the simulation program KinSim (Barshop *et al.*, 1983) using the proposed catalytic mechanism (Scheme 1) and previously determined kinetic parameters for *p*-nitrophenyl ester hydrolysis (Benkovic *et al.*, 1990). At pH 5.9 and 25 °C, these simulations predict that the acyl species comprises nearly 4% of the total antibody concentration during the steady-state period. This calculated value is in reasonable agreement with our measured value of *ca.* 8% acylation.

DISCUSSION

Using electrospray mass spectrometry, we have directly identified a species in which the substrate acyl group is covalently bonded to an active site residue. This identification is based on several lines of evidence. First, in the presence of *p*-nitrophenyl ester substrate, a minor higher mass species is observed in the mass spectrum of the scFv. The observed increase in mass (247 Da) corresponds to the mass of the substrate acyl group (**3**). The acyl group is covalently attached to the scFv since the peak is unperturbed by declustering voltages sufficient to completely dissociate scFv complexes with the hapten [$K_D \leq 1$ nM (Stewart *et al.*, 1994)] or the acid product [$K_D = 20$ μM (Benkovic *et al.*, 1990)]. The acyl group is linked to an active site residue since the formation of the intermediate is blocked by preincubating the scFv with hapten. Finally, replacement of His L91 by Gln (a mutation which destroys catalytic activity) eliminates accumulation of the acylated species. Although these experiments did not allow the direct observation of the hydrolysis products, the collective evidence supports this as a covalent species on the hydrolytic reaction path.

Our results suggest that the NPN43C9 nucleophile is relatively acidic. The observed fraction of acyl–scFv fragment (8%) at pH 5.9 sets an absolute upper limit on the nucleophile pK_a of 7.0; however, since the rate-limiting step in catalysis at low pH is deacylation (Benkovic *et al.*, 1990), this estimate is probably substantially too high. In fact, since the observed concentration of intermediate at pH 5.9 is similar to the calculated steady-state concentration derived from simulations using kinetic parameters obtained at pH 7.0, the nucleophile must be predominantly unprotonated in the pH range 5.9 −7.0 (i.e., the pK_a of the nucleophile is $\leq$5.9). This conclusion is interesting in light of recent proposals that the pK_a of the L91 His side chain is substantially decreased by electrostatic interactions with an arginine group at the active site (Roberts *et al.*, 1994).

Although the existence of acylated intermediates has been proposed from previous kinetic studies of two hydrolytic antibodies (Benkovic *et al.*, 1990; Guo *et al.*, 1994) and an acyl-transfer antibody (Wirsching *et al.*, 1991), this report describes the first physical detection of an acylated form of a catalytic antibody. When considered in the context of previous studies of NPN43C9, our data strongly suggests that the acyl–Fv species is a covalent intermediate on the catalytic reaction pathway of this antibody. The existence of the acyl–antibody intermediate is significant because it directly demonstrates that artificially selected protein catalysts can function using sophisticated mechanisms which are similar to those of naturally evolved enzymes. Transition-state binding interactions may not always be sufficient for facile catalysis; like natural enzymes, efficient catalytic antibodies promote catalysis by providing intrinsic reactive groups at the active site.

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