Efficient site specific removal of a C-terminal FLAG fusion from a Fab’ using copper(II) ion catalysed protein cleavage

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The peptide sequence NKTHC was investigated as a site for efficient, specific cleavage of a fusion protein by cupric ions using a humanised γl Fab’ as a model protein. The native upper hinge NKTHC sequence was mutated to create a site resistant to cleavage by cupric ions and a NKTHC sequence introduced between the hinge and a C-terminal FLAG peptide. Incubation of Fab’ with Cu²⁺ at 62°C at alkaline pHs resulted in removal of the FLAG peptide with efficiencies of up to 86%. Cleavage conditions did not detrimentally affect the Fab’ protein. Use of the NKTHC sequence along with cupric ions may provide a cost-effective method for large scale proteolytic cleavage of fusion proteins.

Keywords: copper (II)/cupric/Fab’/FLAG peptide/protein cleavage

Introduction

In order to aid intracellular solubility, purification and detection, many proteins of biotechnological interest are expressed as fusion proteins including β-galactosidase, IgG binding peptide, glutathione S-transferase, maltose binding protein, His-tag, myc-tag, cellulose binding domain, calmodulin binding peptide, FLAG-tag and Strep-tag (see Ford et al., 1991 for review; Nilsson et al., 1987; Drehner et al., 1991; StoFko-Hahn et al., 1992; Schmidt and Skerra, 1994). If the fusion partner remains associated with the protein of interest after purification it may interfere with protein function, structural analysis, or be immunogenic if the protein is administered in vivo. Removal is therefore desirable. Peptide sites cleaved by specific proteases such as factor Xa, TEV, genenase I, thrombin, enterokinase and 3C can be introduced between two protein domains to enable cleavage of the two domains (Nagai and Thøgersen, 1984; Dougherty et al., 1988; Carter and Wells, 1987; McKenzie et al., 1991; LaVallie et al., 1993; Walker et al., 1994). Such enzymes can be expensive and have variable effectiveness due to differences in sequences surrounding the target site and accessibility of the site to a large enzyme. Their use also requires a further purification step to remove the proteolytic enzyme from the sample.

The upper hinge tetrapeptide sequence NKTHC was identified as the site of cleavage of a highly purified recombinant humanised γl antibody by traces of Cu²⁺. The sequence was cleaved between the Lys and Thr residues. The degree of cleavage increased with increasing temperature, length of time and pH of incubation, and also with increasing amounts of available Cu²⁺ ions. The NKTHC sequence was also cleaved by Cu²⁺ when present in a peptide (Smith et al., 1996).

We investigate the use of Cu²⁺ as an alternative to enzymatic cleavage of fusion proteins, using a humanised γl Fab’ with a C-terminal FLAG peptide as a model protein. A NKTHC site was introduced between the hinge and the FLAG peptide, after mutagenesis of the native NKTHC sequence in the upper hinge to create a ‘null’ site. Dimerization of the Fab’ to F(ab’)₂ via the hinge cysteines, followed by incubation at 59–62°C, pH 9.0 for 8–28 h demonstrated site-specific cleavage and loss of the FLAG peptide. F(ab’)₂ remained in the dimeric state, proving that the ‘null’ site was not cleaved by Cu²⁺. Treatment with Cu²⁺ did not affect the protein since it was intact and retained antigen binding ability of F(ab’)₂.

Materials and methods

Construction of ‘Null 2 FLAG’ expression plasmid

Plasmid pDPH76 encoding Fab 40.4 hinge 1 Δ inter 2 FLAG was derived from plasmid pDPH40 which encodes Fab 40.4 hinge 1 Δ inter 2. pDPH40 has a SpeI restriction site introduced at the 3’ end of the heavy chain (HC) coding region, where the interchain disulphide Cys had been mutated to a Ser, along with an adjacent Ser to Thr change (Humphreys et al., 1997). This enabled cloning of the region encoding the hinge, Cu²⁺ site, spacer and FLAG as an Spel–EcoRI oligonucleotide cassette after annealing the oligonucleotides 5’-CTAGTCTGCAGTGTCCTAGCTGCCTGGCGTGTCCGGAGATCCCATATCCATCAGGAGCGATCTAGCGATTATAAAGATGATGATGAAATGATGAGATTCAACAGTCTCGGACGCCG-3’ and 5’-AACTGCGCGCGCAAGCTTGGATCCATCTATTATCATATCATATCTTATATATCGTAGTACTCCTCGATGTGATTGTTTATCGCGGACGCGG-5’-GCCGTCGACCGAATTGACAGTA-3’. The sequence of the oligonucleotide encoded region was confirmed by sequencing both strands using a PRISM cycle sequencing kit and an ABI 373A sequencing machine.

Production and purification of ‘Null 2 FLAG’ F(ab’)₂

High density bacterial fermentations of strain W3110 bearing pDPH76 were performed as described previously (Humphreys et al., 1997). Extraction of periplasmic material, Protein G purification of Fab’, production and phenyl Sepharose purification of F(ab’)₂ were performed as described previously (Humphreys et al., 1998). F(ab’)₂ protein was concentrated and buffer exchanged with several volumes of PBS using an Amicon pressurised stirred cell with a 10 kDa cut-off membrane to concentrations between 0.5 and 1.6 mg ml⁻¹, and sterilized with a 0.22 μm filter for storage at 4°C.

Cu²⁺ cleavage reactions

F(ab’)₂ was at 0.33 mg ml⁻¹ in all reactions, Tris and other buffers were used at a final concentration of 50 mM (from 500 mM stock solutions). Tris was used at pH 9.0 unless otherwise stated. All metal ions tested were chloride salts from stocks for each concentration tested dissolved in dH₂O.
Reaction volumes were most commonly 15 µl in 500 µl Eppendorf tubes, but were also scaled up to 150 µl. To eliminate evaporation during lengthy high temperature incubations (59–64°C) reactions were layered in paraffin oil. Incubations at 62°C were in an air incubator, whilst other temperatures were in a Biometra TRIO-Thermodblock with heated lids. Samples (1 µg) were withdrawn through the paraffin oil for immediate SDS–PAGE or into 10 mM EDTA and stored at −20°C during time course experiments. Samples for HPLC analysis had paraffin oil removed by pipetting and ether extraction. EDTA-free Complete™ protease inhibitor (Boehringer Mannheim, UK) was used as per manufacturer's instructions.

**SDS–PAGE analysis of 'Null 2 FLAG' cleavage by Cu²⁺**

One µg per lane of Cu²⁺ treated samples were electrophoresed on 4–20% Tris–glycine gels (Novex, UK) after boiling in non-reducing SDS–PAGE loading buffer for 3 min. Gels were stained for 10 min with Coomassie brilliant blue, and destained before analysis by laser scanning densitometry on a Molecular Dynamics model 300A machine using ImageQuant software version 4.2 or drying between cellulose membranes for storage (Novex, UK).

After measurement of the area of absorbance for each of the peaks for the three species, percent total cleavage was calculated thus:

\[
\frac{\text{[absorbance (U) + absorbance (S) + absorbance (D)]}}{\text{[absorbance (S) + absorbance (D)]}} \times 100
\]

Gels were silver stained with 'Silver Stain II' from Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan. Western blotting was performed as described previously (Humphreys et al., 1997), except that the primary antibody used was anti-FLAG monoclonal M2 at 1/500 dilution (Kodak, UK), revealed with ExtrAvidin-HRP (Sigma) at 1/500. ‘Rainbow’ markers were used as molecular weight standards throughout (Novex, UK).

**Surface plasmon resonance**

Kinetic analysis to determine the on and off rates for antigen binding to 'Null 2 FLAG' F(ab)²₂ molecules was performed using a BIACORE 2000 (Biacore AB). ‘Null 2 FLAG’ F(ab)² molecules were captured by an anti-human IgG, which is immobilized on the sensor chip surface, followed by an injection of soluble antigen. Affimipure goat anti-human Ig, F(ab)²₂ fragment specific (Jackson ImmunoResearch) was immobilized on a sensor chip CM5 via amine coupling chemistry to a level of 15 500 RU. HBS buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20; Biacore AB) was used as the running buffer with a flow rate of 10 µl/min. ‘Null 2 FLAG’ F(ab)²₂ was captured by the immobilized anti-human IgG to a level between 180-2400 RU. The sensorgram was regenerated with 2 µl of 30 mM HCl. The sensorgram, due to the Cu²⁺–soluble antigen interaction ensure that true affinity measurements for soluble antigen are made during surface plasmon resonance experiments.

**Mass spectrometry**

Molecular mass for Fab' and F(ab')₂ was determined using Micromass VG Quattro 1 triple quadrupole equipment in positive ion electrospray ionisation mode. F(ab')₂ samples were desalted to remove Tris by multiple volume exchanges with 10 mM ammonium acetate using Microcon concentrators with a 10 kDa membrane cut-off size (Amicon, UK).

**Amino acid composition analysis**

This was carried out at the Department of Molecular and Cellular Biology, University of Aberdeen, UK on a ABI 420A machine after hydrolysis with 6 M HCl for 40 min at 160°C under Argon.

**HPLC purification and N-terminal sequencing of cleaved FLAG tail from F(ab')₂**

**Peptide sequencing**

Peptides in fractions were bound to polyvinylidene difluoride membrane (Prosorb, Applied Biosystems), and then 100 µg polybrene (Biobrene, Applied Biosystems) air-dried onto the membrane. These were then analysed by sequencing on an Applied Biosystems 470A with on-line 120A analyser.

**HPLC and mass spectrometry analysis of peptide cleavage by Cu²⁺**

Peptides dissolved in dH₂O were analysed at 0.5 mM final concentrations in incubations with 50 mM Tris–HCl pH 9.0, and CuCl₂ from 2.5 mM to 100 µM. Reaction volumes of 20 µl were overlayed with paraffin oil and incubated at 62°C for 16–24 h. Paraffin oil was removed by pipetting and ether extraction. Cleaved peptides were purified by HPLC, using a Symmetry C18 column (3.5 micron, 4.6×150 mm) on a Hewlett Packard HP1090 with on-line diode array for spectral analysis. Components were resolved with a linear gradient of 2 to 95% acetonitrile in water/triflouracetic acid (0.1% v/v), at a flow rate of 0.5 ml/min, at 21°C. Peaks absorbing at 214 nm were collected and concentrated under vacuum to remove acetonitrile for peptide sequencing.

**Results**

**Engineering of a Fab' with a C-terminal Cu²⁺ cleavage site**

The Cu²⁺ cleavable Fab' was derived from a previously described humanised γ1 Fab' (‘Fab 40.4 hinge 1 Δ inter’) which binds a human cytokine (Humphreys et al., 1997). The ‘Fab 40.4 hinge 1 Δ inter’ has two γ' hinge cysteines and lacks the inter light chain–Fd disulphide bond (hereafter, interchain), due to Cys→Ser mutagenesis of the interchain residues.

All four residues in the native DKTHC sequence of the upper hinge of ‘Fab 40.4 hinge 1 Δ inter’ were mutated in order to destroy the Cu²⁺ cleavage site, resulting in the protein termed ‘Null 2 FLAG’. Following the middle hinge there is an introduced Cu²⁺ cleavage site DKTHC, a short hydrophilic/
Copper catalysed cleavage of protein

Fig. 1. Demonstration of cleavage of FLAG tail and effect of [Cu^{2+}]. Non-reducing SDS–PAGE using 4–20% Tris–glycine gels. The size and migration of molecular weight markers are shown on the left in kDa. All lanes contain 1 µg protein equivalents (unless indicated) from cleavage reactions using 50 mM Tris buffer at pH 9.0 (except MES which was pH 5.5) for 24 h at 62°C. Lanes 1 to 8 are Coomassie stained. The cleavage reactions in lanes 1–5 were Tris buffered with 5, 2.5, 1, 0.1 mM and 10 µM Cu^{2+} respectively; lane 6 was MES buffered with 1 mM Cu^{2+}; lane 7 was Tris buffered with 10 mM EDTA; lane 8 was Tris buffered with 2.5 mM Cu^{2+} and Complete™ protease inhibitor. Lanes 9–11 show immunodetection of 10 ng equivalent protein loadings with a anti-FLAG antibody. Cleavage reactions were Tris buffered and contain 2.5 mM Cu^{2+} respectively; lane 6 was MES buffered with 1 mM Cu^{2+}; lane 7 was Tris buffered with 10 mM EDTA; lane 8 was Tris buffered with 2.5 mM Cu^{2+} and Complete™ protease inhibitor. Lanes 9–11 show immunodetection of 10 ng equivalent protein loadings with a anti-FLAG antibody. Cleavage reactions were Tris buffered and contain 2.5 mM Cu^{2+} respectively; lane 6 was MES buffered with 1 mM Cu^{2+}; lane 7 was Tris buffered with 10 mM EDTA; lane 8 was Tris buffered with 2.5 mM Cu^{2+} and Complete™ protease inhibitor. Lanes 9–11 show immunodetection of 10 ng equivalent protein loadings with a anti-FLAG antibody. Cleavage reactions were Tris buffered and contain 2.5 mM Cu^{2+} respectively; lane 6 was MES buffered with 1 mM Cu^{2+}; lane 7 was Tris buffered with 10 mM EDTA; lane 8 was Tris buffered with 2.5 mM Cu^{2+} and Complete™ protease inhibitor. Lanes 9–11 show immunodetection of 10 ng equivalent protein loadings with a anti-FLAG antibody.

The lower band representing doubly cleaved species (D) is not immunoreactive to an anti-FLAG antibody, demonstrating that both FLAG tails have been removed from this species (Figure 1, lanes 9 and 10). F(ab')2 treated with 10 mM EDTA shows only a single band of uncleaved material by immuno-detection even after a 24 h incubation at 62°C (Figure 1, lane 11). We believe that the band of ~28 kDa in lane 11 is free HC liberated by breakage of the hinge disulphides by treatment with alkaline EDTA. Cleaved FLAG tail is not immunoreactive in lanes 9 and 10, presumably due to its low abundance and loss of the small peptide during electrophoretic transfer. However, liberated FLAG tail could be visualized after silver staining of more heavily loaded SDS–PAGE (Figure 1, band F lane 12).

Effect of buffer composition and pH

It was known that cleavage of the native IgG upper hinge site was favoured by alkaline pH (Smith et al., 1996). Strong buffering was needed to enable investigation of the pH optimum since concentrated CuCl2 is acidic. Buffers with primary amines are known to have a Cu^{2+} binding capacity (Dawson et al., 1986). We tested the efficiency of cleavage using 50 mM Tris, Bicine, CHES and HEPES as buffers at pH 8.5 using Cu^{2+} concentrations of 1 mM and 100 µM. Surprisingly, Tris resulted in the most efficient cleavage. Bicine and Tricine minimal cleavage, whilst CHES and HEPES resulted in loss of F(ab')2 material (data not shown). The Cu^{2+} binding capacity of the Tris buffer (which contains 1° amines) may actually be beneficial, acting as a Cu^{2+} chelation/buffer system.

Tris was then tested at pH 8.0, 8.5, 9.0, 9.25, and 9.5 to define the optimal pH for cleavage reactions. The results in Figure 2 show clearly an increase in protein cleavage with increasing pH. pH 9.0 was used for all subsequent cleavage reactions as a compromise between efficient cleavage, effective buffering, and the desire to use more neutral incubation conditions. Incubation with 2.5 mM Cu^{2+} at pH 5.5 in MES buffer did not result in cleavage of the FLAG tail (Figure 1, lane 6).

flexible spacer region, and finally a FLAG peptide (Brizzard et al., 1994). The C-terminus sequences of the two heavy chains are shown below for comparison.

`Fab 40.4 hinge 1 Δ inter’ heavy chain
N_{EPKTSDKTHTCPPCPA} C
Cu^{2+}
`Fab 40.4 hinge 1 Δ inter Null 2 FLAG’ heavy chain
N_{EPKTSLQVI}TCCPCDKHTIEGSTSDYKDDDK C
Null2 Cu^{2+} FLAG

‘Null 2 FLAG’ Fab purified by Protein G chromatography from E.coli fermentation cell pastes was found to be intact by mass spectrometry. The observed masses for light chain (LC) and heavy chain (HC) were 23 382.66 ± 1.18 and 26 739.62 ± 1.87 respectively, compared to the predicted masses of 23 384.25 and 26 742.04 respectively. Hence, the introduced Cu^{2+} site, flexible spacer, and FLAG peptide were not cleaved by E.coli proteases or media components.

The Fab’ was dimerized to F(ab')2 to enable demonstration that the mutated native (‘null’) Cu^{2+} cleavage site was not cleaved by Cu^{2+}. ‘Null 2 FLAG’ F(ab')2 lacks the interchain disulphide bond and so separates into di-HC and LC (bands U and L, Figure 1) species during non-reducing SDS–PAGE. Di-HC (∼50 kDa) can remain dimeric only if the ‘null’ site is not cleaved by Cu^{2+}. Since each F(ab')2 molecule contains two Cu^{2+} cleavage sites, three species are possible after incubation with Cu^{2+}: uncut (U), singly cleaved (S) and doubly cleaved (D) (Figure 1). Species with FLAG tails removed migrate more rapidly than uncleaved species during SDS–PAGE. The lower band representing doubly cleaved species (D) is not immunoreactive to an anti-FLAG antibody, demonstrating that both FLAG tails have been removed from this species (Figure 1, lanes 9 and 10). F(ab')2 treated with 10 mM EDTA shows only a single band of uncleaved material by immuno-detection even after a 24 h incubation at 62°C (Figure 1, lane 11). We believe that the band of ~28 kDa in lane 11 is free HC liberated by breakage of the hinge disulphides by treatment with alkaline EDTA. Cleaved FLAG tail is not immunoreactive in lanes 9 and 10, presumably due to its low abundance and loss of the small peptide during electrophoretic transfer. However, liberated FLAG tail could be visualized after silver staining of more heavily loaded SDS–PAGE (Figure 1, band F lane 12).

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Effect of Cu\textsuperscript{2+} concentration

Maximum protein and peptide cleavage was found previously to be at a molar ratio of between 0.5 and 1 Cu\textsuperscript{2+}:protein peptide in a non-Tris buffer (Allen and Campbell 1996; Smith et al., 1996). Since Tris buffer has a significant Cu\textsuperscript{2+} binding capacity, prediction of the effective maximum [Cu\textsuperscript{2+}] was difficult. Hence, the [Cu\textsuperscript{2+}] giving optimal protein cleavage was determined empirically. A range of Cu\textsuperscript{2+} concentrations from 10 mM to 10 \(\mu\)M were tested. 10 and 7.5 mM Cu\textsuperscript{2+} were found to cause immediate and overnight precipitation respectively. SDS–PAGE analysis for the remainder of the titration are shown in Figure 1 and quantification of the effect of [Cu\textsuperscript{2+}] is shown in Figure 3. This demonstrates a clear dependence of the extent of cleavage upon [Cu\textsuperscript{2+}]. Such cleavage was completely inhibited by inclusion of EDTA at a final concentration of 10 mM (lane 7, Figure 1). The extent of cleavage with 2.5 mM Cu\textsuperscript{2+} was unaffected by inclusion of a broad range of protease inhibitors (Figure 1, lane 8), suggesting that this highly purified F(ab\textsuperscript{9})\textsubscript{2} sample was not being subjected to the effects of a contaminating protease. No cleavage of ‘Null 2 FLAG’ is seen after 24 h incubation with Ca\textsuperscript{2+}, Fe\textsuperscript{3+}, Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Ni\textsuperscript{2+} or Zn\textsuperscript{2+} ions at 1 mM (data not shown), demonstrating that the cleavage event was due specifically to the presence of Cu\textsuperscript{2+} ions.

Effect of duration and temperature of incubation

The dynamics of cleavage with 2.5 mM Cu\textsuperscript{2+} are non-linear (Figure 4). There is a short lag phase of approximately 2 h before measurable protein cleavage was observed. Cleavage proceeded fairly linearly until approximately 12–14 h (~50% cleavage), followed thereafter by a slowing in the accumulation of cleaved species. After an overnight incubation with 2.5 mM Cu\textsuperscript{2+} approximately 53% of Cu\textsuperscript{2+} sites had been cleaved, 77% after a 24 h incubation, and reaching 86% after 28.5 h at 62°C. The lag phase may be an indicator of a Cu\textsuperscript{2+} availability effect, or generation of reactive peptide cleaving species (Garrison, 1968; Wolff et al., 1986; Allen and Campbell, 1996).

Increasing the temperature of incubation increases both the initial rate of accumulation of cleaved species and the final percent cleavage (Figure 5). However, at 64°C protein loss was observed during the incubation, resulting in a sharp decrease in cleaved species and a concomitant increase in measurement errors and also loss of LC. We observe a small but significant loss (~25%) of ‘Null 2 FLAG’ at 62°C toward the end of the incubation. Incubation at 59°C for 28.5 h results in 71% cleavage and no loss of ‘Null 2 FLAG’.

Specificity of Cu\textsuperscript{2+} catalysed protein cleavage

Electrospray mass spectrometry of a sample of ‘Null 2 FLAG’ cleaved with 2.5 mM Cu\textsuperscript{2+} for 18 h was used to investigate whether ‘Null 2 FLAG’ was being cleaved as expected between the Lys and Thr of the introduced NDKTHC sequence. Depending upon the extent of cleavage each di-HC species can have one or both FLAG tails removed, resulting in species S and D respectively (Figure 1). Cleavage between Lys and Thr results in species with predicted masses of 51 573.35 and 49 664.62 for the singly and doubly cleaved species respectively. We find that the two strongest peaks relating to di-HC had observed masses of 51 565.68 ± 10.47 and 49 662.08 ± 11.16, which are in agreement with the predicted masses for di-HC with one and two FLAG tails removed respectively. In addition, we find no evidence of the species that would be generated if the di-HC were cleaved at the ‘Null 2’ site, nor for non-specific Cu\textsuperscript{2+} cleavage fragments.

N-terminal amino acid sequencing of HPLC purified Cu\textsuperscript{2+}cleaved FLAG tail was performed to ascertain the accuracy of cleavage, and integrity of the cleavage site amino acids.
The most abundant fraction at the start of the elution gradient was collected and gave complete sequence of αHTHIEGSTS-DYKDDDDK²C, confirming accurate cleavage at the predicted site, and that the Thr and His residues in the Cu²⁺ site had not been destroyed or modified during the reaction.

To confirm that the ‘Null 2’ sequence was not cleavable we tested the effect of incubation of the peptides ‘NULL 2’ (N°C-VEPKTLSQVLT-NH₂²C) and ‘CUT 1’ (N°C-VEPKTSĐTHT-NH₂²C) with Cu²⁺ in 50 mM Tris-HCl pH 9.0 at 62°C. The peptide ‘NULL 2’ was not cleaved by Cu²⁺ from 0.1 to 2.5 mM (data not shown), whereas ‘CUT 1’ was cleaved by the higher concentration of Cu²⁺. After incubation of CUT1 with 2.5 mM Cu²⁺, collection of peaks from HPLC purification, electrospray mass spectrometry showed the presence of a major and a trace species with molecular masses of 944.5 and 1281.8 respectively. CUT1 peptide cleaved between the Lys and Thr of the cleavage site (Ac-VEPKTSĐKTH-NH₂²C) has a predicted mass of 944.48, whilst the full length and unmodified CUT1 peptide has a predicted mass of 1282.5. Hence the CUT1 peptide is correctly cleaved between the Lys and Thr residues.

**Table I.** Effect of incubation conditions on affinity of ‘Null 2 FLAG’ F(ab)²

<table>
<thead>
<tr>
<th>Treatment</th>
<th>kₐ (Ms⁻¹)</th>
<th>k₈ (s⁻¹)</th>
<th>K₈ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl pH 9.0, 62°C, 16 h, 2.5 mM Cu²⁺</td>
<td>4.22 × 10⁶</td>
<td>2.08 × 10⁻⁴</td>
<td>4.93 × 10⁻⁴</td>
</tr>
<tr>
<td>50 mM Tris-HCl pH 9.0, 62°C, 16 h, 10 mM EDTA</td>
<td>4.27 × 10⁶</td>
<td>1.85 × 10⁻⁴</td>
<td>4.33 × 10⁻⁴</td>
</tr>
<tr>
<td>Untreated</td>
<td>3.93 × 10⁶</td>
<td>1.28 × 10⁻⁴</td>
<td>3.26 × 10⁻⁴</td>
</tr>
</tbody>
</table>

**Effect of cleavage conditions on protein function**

SDS-PAGE and mass spectrometry analysis showed that the cleavage conditions had not grossly affected the integrity of the ‘Null 2 FLAG’ protein, as witnessed by the absence of new protein fragments. We took advantage of the antigen binding ability of the Fab 40.4 derived ‘Null 2 FLAG’ to investigate whether more subtle changes in the protein occur after cleavage reaction incubation. Modification of residues in the CDRs might well result in a loss of antigen binding. Gly, Pro, Lys and His in particular are known to be sites for protein damage by divalent metal ions (Dean et al., 1989; Hawkins and Davies, 1997; Easton et al., 1997). The CDRs of ‘Null 2 FLAG’ contain a total of five Gly, two Lys and one Pro residues, so we might expect that any non-specific damage to the F(ab)² might be reflected more measurably in loss of affinity due to damage specifically to the CDRs. Surface plasmon resonance affinity measurements (Table I) show that overnight incubation at pH 9.0 with or without 2.5 mM Cu²⁺ do not significantly affect the affinity of ‘Null 2 FLAG’ F(ab)². The minor apparent loss of affinity is actually caused mainly by the incubation procedure, and not due to the Cu²⁺ itself. This may be a reflection of the ability of the LC and HC of the F(ab)² to part, due to the absence of the interchain disulphide bond. Use of a capturing antibody in the surface plasmon resonance experiments demonstrates that the epitopes recognized by this polyclonal anti-sera on ‘Null 2 FLAG’ F(ab)² are structurally intact also.

In addition, we performed a total amino acid composition analysis on ‘Null 2 FLAG’ F(ab)² that had been incubated for 16 h at 62°C in 50 mM Tris pH 9.0 with or without 2.5 mM Cu²⁺. There were no new peaks in the Cu²⁺ treated sample that might be evidence of modified amino acids, and no significant difference in the amino acid composition between the two samples that might be evidence of degradation of particular amino acids (I. Davidson, University of Aberdeen, personal communication).

**Discussion**

We show evidence for use of the tetra-peptide sequence ⁸DKTH⁹F as a site for specific cleavage of fusion proteins by cupric ions. In our model protein a C-terminal FLAG peptide is cleaved off exactly between the Lys and Thr of the target sequence as shown by mass spectrometry of the remaining N-terminal F(ab)² part, and N-terminal sequencing of liberated C-terminal FLAG peptide.

The cleavage is efficient, reaching levels of 71, 81 and 86% after 28.5 h incubations at pH 9.0, and 59, 61 and 62°C respectively. The cleavage site does not need to be dimeric in order to be cleaved, since we see significant accumulation of F(ab)² species both with one and two FLAG tails removed. We find no evidence for gross deleterious effects on the structure or activity of the F(ab)² used in this system, even after incubation with 2.5 mM Cu²⁺ for 16 h at 62°C, pH 9.0. The effective pH in each ‘pH 9.0’ cleavage reaction is actually approximately pH 7.9 due to the inverse temperature dependence of Tris pH control (Dawson et al., 1986). So, the pH conditions are less harsh than they might initially appear. It is clear that the high incubation temperature is the most critical element in determining the quality of protein after the cleavage treatment. With this particular protein the 5°C difference between 59 and 64°C changes the incubation from one that gives a yield of 71% cleavage and unmeasurable protein loss to one with ≈70% protein loss after a 28.5 h incubation. However, the maximum tolerated temperature for other proteins needs to be determined empirically.

The efficiency of cleavage is high even without final optimization of the cleavage process for ‘Null 2 FLAG’ F(ab)². Simply by increasing the [Cu²⁺] to greater than 2.5 mM, and the pH to 9.5 would increase the extent of cleavage at any given time. Another possible method for increasing the speed of cleavage is to optimize the cleavage site itself, and/or the micro-environment in which it is founded. Others (Allen and Campbell, 1996) have shown with a model peptide that changing the Thr of the ⁸DKTH⁹F sequence to Ser effected a 78% increase in cleavage efficiency. It would be interesting to see whether such benefits can be transferred to our fusion protein model. In addition, conservative changes in the other amino acids of the ⁸DKTH⁹F sequence (e.g. D→E, K→R and H→Y) might effect additional changes in rate or metal ion specificity of cleavage (Hawkins and Davies, 1997; Cooper et al., 1985).

The amino acids immediately around the ⁸DKTH⁹F sequence may be important in non-specifically chelating Cu²⁺ to the local environment of the cleavage site. Acidic residues may stabilize the postively charged Cu²⁺ ion, and Cys, Met and His which are known to be involved and copper chelation in proteins may help stabilize/orientate the ion (Adman, 1991).
In addition, study of peptide cleavage conditions suggest that a denatured state may enable efficient cleavage of the $^{\text{NDKTH}}_{\text{C}}$ sequence (Allen and Campbell, 1996).

The mechanism of Cu$^{2+}$ catalysed cleavage of the $^{\text{NDKTH}}_{\text{C}}$ sequence is not known. It possibly involves Cu$^{2+}$ catalysed site specific conversion of O$_2$− radicals to OH radicals which cause localized cleavage of the peptide bond. Metal ion catalysed reactions involving amino-acid chelation sites can result in destruction of the amino-acid, such as His→Asp conversions. However, here neither the T or H of the $^{\text{NDKTH}}_{\text{C}}$ site are destroyed during the cleavage (for review see Wolff et al., 1986; Hawkins and Davies 1997; Cooper et al., 1985; Marx and Chevion, 1985).

The ‘Null’ site of ‘Null 2 FLAG’ contains changes to all four residues ($^{\text{NDKTH}}_{\text{C}}$→$^{\text{NQVL}}_{\text{C}}$). To minimize the risk of immunogenicity against Fab' material cleaved by this technique requires making the least number of changes in the native $^{\text{NDKTH}}_{\text{C}}$ site whilst remaining uncleavable by Cu$^{2+}$. The same four residues (D, K, T and H) are collectively involved in the metal binding site of serum albumins, and so are clearly capable of chelating divalent cations (Sadler et al., 1994). The pH dependence of the cleavage reaction and peptide variants implicate the importance of the His residue in the cleavage reaction (Allen and Campbell, 1996). Mutation of this residue alone, or combined with mutation of the Thr residue to a small or hydrophobic residue might be sufficient to reduce the potential immunogenicity.

We find no evidence for cleavage of ‘Null 2 FLAG’ F(ab’)$_2$ at sites other than that of the $^{\text{NDKTH}}_{\text{C}}$ sequence, the tetrapeptide is absent from the 1st structure of ‘Fab 40.4’. The $^{\text{NDKTH}}_{\text{C}}$ sequence is also relatively rare elsewhere. Only 167 matches are found against 256 226 entries searched in the NCBI protein database. To be cleaved efficiently by Cu$^{2+}$ this sequence must have an exposed and probably disordered structure. This combination of rarity of both 1st and 3rd structure of the Cu$^{2+}$ cleavage site probably makes the target specific enough to be a general protein cleavage site. The fusion protein retains two amino acids of the site after cleavage. This may only be of importance where potential immunogenicity is an issue.

The enormous benefit of using $^{\text{NDKTH}}_{\text{C}}$/Cu$^{2+}$ is in cost and convenience. Many proteases cannot be used for large or industrial scale processes due to the cost involved, and the problem of ensuring complete removal of contaminating protease after cleavage. Assuming equal cleavage efficiencies, the cleavage of protein with CuCl$_2$ is approximately 10$^2$-fold cheaper than using Factor Xa protease. Mineral derived CuCl$_2$ has no risk of viral/prion contamination that may be found when using some proteases. It can be removed simply from the protein sample by addition of EDTA, followed by dialysis. When the fusion partner is an affinity tail cleaved material should be in the flow through after re-loading onto an affinity column, giving a simple method of separating cleaved and uncleaved material.

In summary, we have demonstrated the efficient and site specific cleavage of the $^{\text{NDKTH}}_{\text{C}}$ sequence by Cu$^{2+}$ to effect the removal of a C-terminal FLAG fusion from a humanised γ' Fab'. We do not know how the cleavage efficiency of $^{\text{NDKTH}}_{\text{C}}$ in this case has been influenced by the surrounding residues that form part of the middle hinge, flexible spacer region, and acidic FLAG peptide. It will be of interest to investigate how robust other proteins will be during prolonged high temperature, alkaline incubations. The simplicity and rarity of this cleavage site could make copper cleavage of protein fusions a widely applicable technique.

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References


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