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Heterologous Overexpression of Human NEFA and Studies on the Two EF-Hand Calcium-Binding Sites

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Human NEFA is an EF-hand, leucine zipper protein containing a signal sequence. To confirm the calcium binding capacity of NEFA, recombinant NEFA analogous to the mature protein and mutants with deletions in the EF-hand domain were expressed in *Pichia pastoris* and secreted into the culture medium at high yield. The calcium binding activity of each purified protein was measured by a modified equilibrium dialysis using the fluorescent Ca^{2+} indicator FURA-2 and atomic absorption spectroscopy. A stoichiometry of 2 mol Ca^{2+}/mol NEFA was determined. The Ca^{2+} binding constants were resolved by intrinsic fluorescence spectroscopy. Fluorescence titration exhibited two classes of Ca^{2+} binding sites with Kd values of 0.08 μM and 0.2 μM. Circular dichroism (CD) spectroscopy showed an increase from 30 to 43% in the amount of α-helix in NEFA after addition of calcium ions. Limited proteolytic digestion indicated a Ca^{2+} dependent conformational change accompanied by an altered accessibility to the enzyme.

NEFA (DNA binding, EF-hand, Acidic amino acid rich region) is a calcium binding EF-hand protein. Human NEFA was originally identified in the common acute lymphoblastic leukemia cell line KM3 and its primary structure was analyzed by cDNA sequencing [1]. The deduced 420 amino acid sequence was confirmed by partial peptide microsequencing of the mature protein isolated from the same cell line [2]. NEFA is a mosaic protein consisting of seven regions. From the N-terminus to the C-terminus, the following characteristic amino acid sequence motifs are aligned: a signal peptide of 25 amino acid residues, a leucine/isoleucine rich region, a basic putative DNA binding domain with a potential bipartite nuclear targeting signal, a first EF-hand site, an acidic region, a second EF-hand site and a leucine-zipper region (Fig. 1). The molecular mass of the mature protein was determined to be 48 kDa by laser desorption mass spectrometry, but its apparent relative molecular mass on SDS–PAGE was 55 kDa [1, 2]. This high value may be caused by the content of 47% charged amino acid residues, because neither N- nor O-glycosylation was detected.

The biological role of NEFA is still unknown, however, it shows strong sequence homology to the 63 kDa protein nucleobindin (Nuc) [3] in the basic region and the EF-hand domain. In contrast, the signal peptide and N-terminal sequences as well as the leucine-zipper and consecutive C-terminal sequences are divergent. Besides, the human NEFA and Nuc proteins are encoded by two separate and unlinked gene loci: the NEFA gene is located on chromosome 11p14-p15 [4] and the Nuc gene on chromosome 19q13.2-13.4 [5]. Although the significance of independent gene regulation and the consequences of the structural differences are so far not understood, we presume that the partial sequence homologies, especially the order and similarity of their structural motifs, indicate related, but not necessarily identical functions of NEFA and Nuc.

Murine Nuc was characterized as a soluble factor that promoted an autoimmune response against single and double stranded DNA in lupus-prone MRL/lpr mice [6,7] and is discussed to accelerate apoptosis [8]. This protein binds Ca^{2+} ions via its EF-hand domain [9] and also binds DNA [3]. While calcium binding could be confirmed by the present work, no DNA binding activity has been observed for NEFA so far. Bovine Nuc was also isolated as a noncollagenous extracellular matrix protein from bone. As to its possible role in bone tissue, it was discussed whether it is involved in osteoid mineralization or in calcium homeostasis [10]. Recently, rat Nuc was identified as a Golgi resident protein that may contribute to calcium homeostasis in the *cis*-Golgi network and *cis*-Golgi cisternae [11]. Nuc protein localized to the Golgi was named CALNUC to differentiate its function and its topography from the previously known Nuc. CALNUC interacted with the

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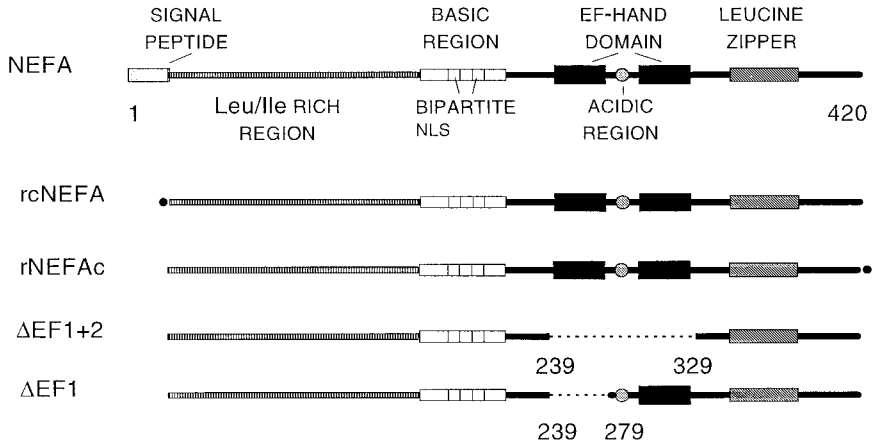

FIG. 1. Schematic representation of NEFA, rcNEFA, rNEFAc, ΔEF1+2, and ΔEF1. The NEFA precursor protein consists of 420 amino acid residues. rcNEFA and rNEFAc are analogous to the mature NEFA protein except for the Cys residues added at the N-terminus and the C-terminus, respectively. ΔEF1+2 lacks both EF-hand sites; ΔEF1 lacks the first EF-hand site. Solid black areas indicate the positions of the two EF-hands, the gray bar the hydrophobic signal peptide. The white area denotes the basic region with the potential bipartite nuclear localization signal sequence (NLS). The internal deletions are characterized by broken lines. The black disks denote the introduced Cys residues, gray disks the acidic region. The black and white line represents the Leu/Ile-rich region. The striped area describes the leucine zipper. The numbers shown correspond to the amino acid positions in the NEFA precursor sequence.

$G\alpha i_3$ subunit of heterotrimeric G proteins, while Nuc was found to interact with $G\alpha i_2$ [12].

Here we present the heterologous overexpression of NEFA and NEFA mutants in the methylotrophic yeast, *Pichia pastoris*. The expression products were used in experiments to verify whether NEFA actually binds Ca^{2+} via its EF-hand domain and whether Ca^{2+} binding induces a conformational change in the molecule.

MATERIALS AND METHODS

Construction of the expression vectors. The heterologous gene expression of NEFA was carried out in the *Pichia pastoris* strains GS115 and SMD1168 (Invitrogen) using the shuttle expression vector pPIC9 (Invitrogen) [13, 14], in which the α-factor prepro leader peptide (MFα) from *Saccharomyces cerevisiae* [15] had been integrated to secrete the expressed protein into the culture medium. NEFA variants were generated from NEFA-cDNA clone IV [1] by PCR [16] using *Vent* polymerase (BioLabs).

The nucleotide sequence in parentheses of (1) and (2) flanking the 5′-end of NEFA is derived from the 3′-end of MFα and encodes a KEX2 endopeptidase cleavage site [17] as well as the employed nuclease recognition site. The underlined nucleotides mark the inserted Cys codons. Variant rcNEFA and rNEFAc are analogous to the mature NEFA protein except for Cys at the N-terminus and the C-terminus, respectively. Primers (1) and (3) were used to construct rcNEFA, and primers (2) and (4) were employed to amplify rNEFAc. Both fragments were inserted into *Xho*I and *Not*I sites of pPIC9. For the construction of variant ΔEF1+2 lacking the two EF-hand sites, a DNA fragment corresponding to the 92 C-terminal amino acid residues of the NEFA protein was amplified with primers (5) and (3) from clone IV. The resulting fragment was then inserted at the *Bam*HI cleavage site corresponding to amino acid positions (a.p.) 239-240 of the NEFA precursor protein and the *Not*I cleavage site in the recombinant pBluescript-NEFA clone SKN. Into this clone, a cDNA fragment corresponding to mature NEFA containing the KEX2 endopeptidase cleavage site nucleotide sequence at the 5′-end of the NEFA cDNA had already been inserted in our laboratory. Finally the *Xho*I/*Not*I fragment of the newly generated clone SKN/ΔEF1+2 was subcloned into pPIC9. ΔEF1, lacking the first EF-hand sequence, was constructed in the same manner, by amplification of a DNA fragment corresponding to the C-terminal 142 amino acid residues, a.p. 279–420, with primers (6) and (3). The DNA sequences of the constructed recombinant plasmids were confirmed on an automatic DNA sequencer ABI PRISM 377 using Big Dye Terminator Chemistry.

Oligonucleotides.

(1) *Xho*pi(F), 5′-(ATCTCTCGAGAAAAGAGAGGCTGAAGCTTAC)-TGTCCTATTGACATAGACAAGAC-3′,
(2) *Xho*pro(F), 5′-(ATCTCTCGAGAAAAGAGAGGCTGAAGCTTAC)-CCTATTGACATAGACAAGAC-3′,
(3) *Not*I(R), 5′-(ATTAGCGGCCGC)TTAAATGTGTGGCTCAAA-CTT-3′,
(4) *Not*ICys(R), 5′-(ATTAGCGGCCGC)TCAACAAATGTGTGGCT-CAAACTTCAA-3′,
(5) *Bam*EF1+2(F), 5′-AATTCTTGGATCCAGATAGC-3′,
(6) *Bam*EF1(F), 5′-GTATAGGATCCTAAAAATGAAGAGGAT-3′.

Transformation and expression. 9 μg of *Bgl*II-cleaved, linearized, recombinant plasmid DNA were used to obtain stable *P. pastoris* strain GS115 transformants by the spheroplast transformation method [13]. Screening for His^+, Mut^- and AOX1-disrupted transformants was performed as described in the Pichia Expression Protocol (Invitrogen). Positive clones were grown for 2 days in 12 ml BMGY, harvested, resuspended in 3 ml expression medium BMMY and further cultured in BMMY for 2 days at 30°C and 220 rpm. 20 μl of the supernatants were analyzed for product by standard SDS–PAGE [18]. The stain representing the highest level of secreted mutant protein was used for intermediate scale expression following the Invitrogen protocol except that in some cases the induction in BMMY medium was carried out for only one day.

Protein purification. Recombinant human NEFA and mutants were precipitated at 50% ammonium sulfate from 400 ml clear culture supernatants. The pellet was dissolved in 8 M urea, 0.01 M Tris/HCl, pH 7.2 and was applied to a 4 × 150 cm Sephacryl S-200 HR column (Pharmacia) in 6 M urea, 0.1 M Tris/HCl, pH 7.5. 5 ml fractions were collected at a flow rate of 30 ml/h. Fractions were analyzed by SDS–PAGE. The relevant fractions were pooled, dialyzed excessively against 0.1 M KCl, 0.01 M Hepes, pH 7.2 and

concentrated by ultrafiltration in Ultrafree-15 Biomax-30 kD centrifugal filter devices (Millipore). For analytical scale purification RP-HPLC [19] was utilized. 75 μg protein in 120 μl of 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA were applied to a Vvdac C4 column (0.4 cm $\times$ 25 cm, MZ) equilibrated with solvent A. Separation was performed at 40°C and a flow rate of 1 ml/min. The following linear gradient was used: 0%-25% solvent B within 20 min, 25%-65% solvent B within 55 min and 65%-100% within another 5 min. Solvent A: 0.1% (v/v) TFA; solvent B: 85% acetonitrile in 0.1% (v/v) TFA.

Protein sequencing and further analysis. Desalted fractions from gel filtration were subjected to SDS–PAGE, stained with Coomassie brilliant blue R-250, destained and electroblotted onto PVDF membranes [20]. The membranes were dried for 48 h under vacuum. For N-terminal sequence analysis [21] NEFA and mutant protein bands were excised and applied to an automated gas phase sequencer model 473A with an online PTH analyzer (Applied Biosystems). For Western blots, protein gels were electroblotted without staining. PVDF membranes were incubated as previously described with the polyclonal Antibody FP12 raised in rabbits against NEFA-GST fusion protein [1]. Protein concentrations were routinely measured by BCA assay [22]. To determine exact concentrations protein samples were hydrolyzed with 6 N HCl and 1% (w/v) Phenol for 1 h at 150°C, and the amino acid composition was analyzed on a Durrum Analyzer D 500 [23].

Removal of contaminating metal ions. To reduce metal contamination, no metal or glassware was used for these studies and the required buffer solutions were routinely passed through a column containing 100 g of Chelex-100 (Bio-Rad). Protein solutions were dialyzed twice in washed polyethylene (PE) cylinders in a volume of 5 l 0.1 M KCl, 0.01 M Hepes pH 7.2 in the presence of 60 g Chelex-100. Dialysis bags (Roth) were boiled for 30 min in 1% sodium bicarbonate solution, followed by washing with distilled water, treatment with 5 mM EGTA and rinsing with Ca^{2+}-free buffer. Cuvettes for spectroscopy were soaked for 2-4 h in 1:1 mixture of 1 M HCl and ethanol, rinsed with deionized water and rinsed several times with metal-free 0.1 M KCl, 0.01 M Hepes pH 7.2. Total Ca^{2+} concentration was determined with a Perkin–Elmer Cetus Instrument 2380 atomic absorption spectrophotometer.

Limited tryptic digestion. Proteolytic fragmentation was performed according to Drabikowski *et al.* [24] with several modifications. Aliquots of 20 μg rcNEFA, ΔEF1+2 and calmodulin from hog brain (Boehringer-Mannheim) in 100 mM Tris/HCl pH 8.5 were digested at 20°C by trypsin (Boehringer-Mannheim) at a ratio of 1:1000 (w/w) in the presence of 1 mM EGTA (Serva), 100 μM $CaCl_2$ or 100 μM $MgCl_2$. The reaction was stopped by the removal of an aliquot every 2 min followed by its immediate denaturation at 100°C in SDS–PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, 2.3% (w/v) SDS, 10% (w/v) Glycerol, 0.1% (w/v) Bromphenol-Blue).

Quantitative analysis of calcium binding. Defined amounts of metal-free protein were added to 1 mM $CaCl_2$ in 0.1 M KCl, 0.01 M Hepes, pH 7.2 in the top chambers of Centricons (Amicon) prewashed with calcium free 0.1 M KCl, 0.01 M Hepes, pH 7.2 and buffer was centrifuged at 3000 $\times$ g for 25 min through the 10-kDa cutoff membrane. Aliquots of the flow-through buffer were added to 0.1 M KCl, 0.01 M Hepes pH 7.2 containing a final concentration of 1 μM ratioable fluorescent Ca^{2+}-indicator FURA-2 [25] (Molecular Probes). The ratio of fluorescence intensity between 340 nm and 380 nm excitation wavelength was monitored at an emission wavelength of 510 nm. Using buffers with known amounts of Ca^{2+} ions and the equation of Tsien [26], it was possible to calculate the Ca^{2+} concentration in the flow-through buffer and thus the amount of Ca^{2+} bound to the protein. For direct binding assays, 2 ml aliquots of 39 μM metal-free protein in standard dialysis bags were incubated at 4°C for 20 h in the above described buffer with 0.05, 0.1 and 3 mM $CaCl_2$. The total amount of Ca^{2+} in the protein-containing samples and the dialysis buffer was determined by atomic absorption spectroscopy.

Calcium titration by intrinsic fluorescence. Fluorescence spectra of 21.6 μM protein solution in 0.1 M KCl, 0.01 M Hepes, pH 7.2 with and without 3 mM Mg^{2+} ions were obtained with a Perkin–Elmer LS 50B luminescence spectrometer over the 300-400 nm wavelength range with 1 cm path-length cells in a volume of 2 ml. The cell was maintained at 20°C with a circulating water bath (LKB). Fluorescence was measured with 2 nm slits in ratio mode 90° to the source. The intrinsic fluorescence of the protein solution was monitored by exciting at 280 nm and monitoring the emission at 335 nm. To monitor changes in the Tyr and Phe environment, spectra were taken after every Ca^{2+} increment. The computer averaged 10 scans and the signal due to solvent was subtracted. Concentration of free Ca^{2+} in solution was controlled by means of an EGTA containing buffer.

Evaluation of titration data. The degree of saturation $Y = (F - F0)/(F\infty - F0)$ was calculated from the fluorescence signal F, the signal at zero calcium F0 and the signal at saturation F∞. The free metal concentration was computed using the program "Calcium" version 2.1, (developed by K. J. Foehr, programmed by W. Warchol) based on the original principles outlined by Perrin and Dempsey [27]. The experimental data were imported in the program "Origin" version 3.0 (Microcal Software, Inc.) and computer-fitted to the following equation, which describes the interaction between two types of binding sites:

$$Y_{fit} = \frac{Y_E + \frac{[L]}{Kd_1} \times Y_{EL} + \frac{[L]^2}{Kd_1 \times Kd_2} \times Y_{EL2}}{1 + \frac{[L]}{Kd_1} + \frac{[L]^2}{Kd_1 \times Kd_2}}$$

Y_E describes the degree of saturation of the protein without the ligand. Y_{EL} denotes the degree of saturation of the complex with one ligand bound and Y_{EL2} indicates the degree of saturation of the complex with two ligands. [L] is the ligand concentration, Kd_1 and Kd_2 the dissociation constants.

Circular dichroism (CD) spectroscopy. CD measurements were performed on a J-720 spectropolarimeter (Jasco) in a volume of 250 μl containing 0.1 μg/μl protein solution in 0.01 M sodium phosphate buffer pH 7.2 at 25°C. Far-UV scans from 255 nm to 195 nm were recorded with 1 nm slits in a resolution of 10 mdeg in the absence of Ca^{2+} (1 mM EGTA) and in the presence of 200 μM $CaCl_2$. The signal due to the solvent was subtracted. After the measurements the protein concentration was determined by amino acid analysis.

RESULTS

Expression of rcNEFA, rNEFAc, and deletion mutants. rcNEFA, rNEFAc and the two deletion mutants ΔEF1+2 and ΔEF1 were expressed by *P. pastoris* strain GS115 and secreted into the cell culture medium. Secretion was controlled by the MFα from *S. cerevisiae* instead of the original NEFA signal sequence. The *P. pastoris* strain GS115 secretes very low levels of its own native yeast proteins (Fig. 2). A high level secretion rate of 100-200 mg protein per liter was achieved in the cleared culture supernatant for rcNEFA, rNEFAc and ΔEF1+2, but for ΔEF1 merely 20 mg per liter were observed. Unfortunately the expressed NEFA variants were found to be partially degraded. This proteolytic processing presumably occurred in the intracellular secretory pathway, as no further proteolysis in the harvested culture supernatant was observed after incubation at room temperature for one day. To reduce the amount of fragments, the protease deficient strain SMD1168, a pep4 mutant strain

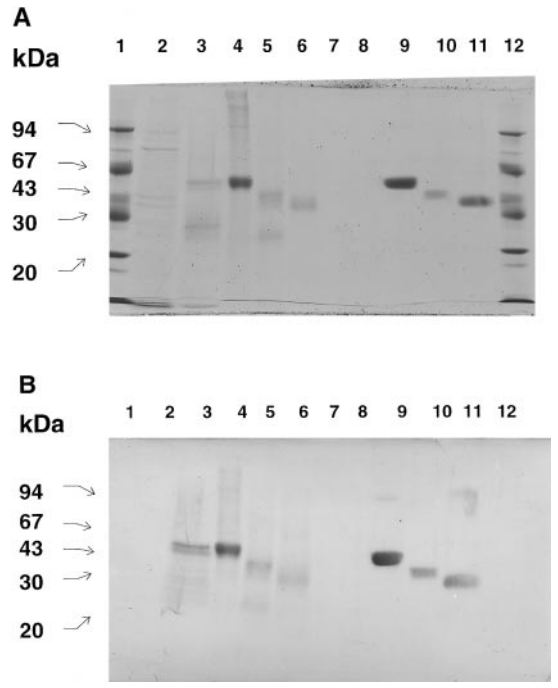

FIG. 2. NEFA variants expressed in *P. pastoris.* (A) SDS–PAGE of protein from cleared culture supernatant and purified protein. Lanes 1 and 12, LMW marker Lane 2, 10 μl of 4 times concentrated culture supernatant of untransformed *GS115;* Lanes 3 and 4, unconcentrated supernatant of rcNEFA and rNEFAc, respectively; Lanes 5 and 6, 5 μl of an ammonium sulfate precipitation of each ΔEF1+2 and ΔEF1. Lanes 7 and 8, empty; Lanes 9-11, about 0.8 μg of purified proteins as obtained by gel filtration and RP-HPLC; Lane 9, rcNEFA; Lane 10, ΔEF1; Lane 11, ΔEF1+2. The gel was stained with Coomassie brilliant blue R-250. (B) Western blot of proteins. After PAGE the proteins were transferred onto PVDF membrane by semidry electroblotting. The membrane was incubated with anti-NEFA rabbit polyclonal antibody FP 12 [1] and detected with the HRP-conjugated anti-rabbit swine IgG antibody. Lanes are analogous to (A).

of *P. pastoris,* was transformed with the construct ΔEF1. Even so, no significant reduction of fragmentation could be detected in the culture medium (data not shown).

Protein purification and analysis. To isolate the intact protein several purification steps were performed: 5 fold concentration of 400 ml cleared culture supernatant, 50% ammonium sulfate precipitation, and chromatography on a Sephacryl S-200 column in 0.01 M Tris/HCl, pH 7.2 and 6 M urea. Thereby relatively pure full length proteins were obtained at a yield of about 10 mg per chromatography run. This grade of purity was found to be sufficient for affinity chromatography and for immunization of rabbits. For quantitative calcium binding assays, further purification was performed by RP-HPLC on a Vydac C4 column. The purified rcNEFA, ΔEF1 and ΔEF1+2 showed an apparent molecular weight of 55 kDa, 50 kDa and 45 kDa, respectively, on SDS–PAGE (Fig. 2). These data correspond to the full length of expected products from each construct. N-terminal amino acid sequencing of the purified proteins was performed by automated gas phase sequencing. The results are listed in Table 1. The N-termini of recombinant NEFA variants were not homogeneous, except for variant ΔEF1. The amino acid sequence of the KEX2 endopeptidase cleavage site, an integrated constituent of the vector, was identified only at the N-terminus of a minor population of rcNEFA. The N-terminus of the major population was Val34 of the NEFA precursor [1]. The recombinant proteins rNEFAc, ΔEF1 and ΔEF1+2 showed Ile27 as the predominant and Val34 as the minor N-terminal amino acid residue (Table 1). The main fraction of the proteolytically degraded fragment of rcNEFA showed a relative molecular weight of 30 kDa on SDS–PAGE (Fig. 2). Its N-terminal amino acid sequence started at Asn196 of the NEFA precursor with the sequence N E E K R K.

Quantitative analysis of calcium binding. The Chelex-100 treated protein solutions of the recombinant NEFA variants contained less than 0.6 μM calcium as determined by atomic absorption spectroscopy. To investigate calcium binding, the quantitative data shown in Table 2 were collected by ultrafiltration using Centricon-10 tubes and by FURA-2 titration of the flow-through buffer. In this assay, rcNEFA bound 1.48 mol Ca^{2+}/mol protein. The commercially available calmodulin used in a control experiment bound 3.18 mol Ca^{2+}/mol protein. According to previous studies, 4 mol Ca^{2+} bind to one mol calmodulin [28, 29]. This aberration may be due to differences in the purification procedures of calmodulin or acquired Ca^{2+} contamination during the experiment. The calcium binding assay indicated NEFA to bind 2 mol of Ca^{2+} ions. In contrast, none of the EF-hand deletion mutants ΔEF1+2 and ΔEF1 showed any detectable calcium binding under

TABLE 1

N-Terminal Amino Acid Sequence of NEFA Variants Compared to Mature NEFA Isolated from KM3 Cells

Protein	N-terminal sequence	Starts at position	Amount
NEFA	PIDIDKTKVQNIH..	26	major
rcNEFA	VQNIH..	34	major
	EAEAYCPIDIDKTKVQNIH..	*26	minor
rNEFAc	IDIDKTKVQNIH..	27	major
	VQNIH..	34	minor
ΔEF1+2	IDIDKTKVQ..	27	major
	VQNIHPVE..	34	minor
ΔEF1	IDIDKTKVQN..	27	major

Note. NEFA: mature NEFA protein from the KM3 cell line. The numbering of amino acid positions corresponds to the NEFA precursor protein [1] (GenBank Accession No. X76732).

* The N-terminal sequence EAEAY of rcNEFA was derived from the KEX2 endopeptidase cleavage site of MFα, involved in the vector pPIC9 and linked to the N-terminus of the expression constructs [14]. The underlined Cys residue was introduced by primer directed mutagenesis.

TABLE 2

Calcium Binding Capacity of rcNEFA, Calmodulin, and NEFA Variants

Protein	Ratio $mol^{2+}_{Ca}/mol_{protein}$
calmodulin	3.18
rcNEFA	1.48
ΔEF1+2	0.26
ΔEF1	0.31

Note. Metal-free proteins were incubated with 0.2 mM $CaCl_2$. The content of unbound Ca^{2+} ions was measured using the fluorescent calcium indicator FURA-2 [25].

the same experimental conditions. The data obtained by the direct Ca^{2+} binding assay employing atomic absorption spectroscopy support these results, indicating binding of 2 Ca^{2+} ions to one NEFA molecule and no binding to the deletion mutants. These results verify that the EF-hand pair configuration cooperatively mediates Ca^{2+}-binding.

Intrinsic fluorescence. Tyrosine/tryptophan fluorescence of rcNEFA was excited at 280 nm and an emission spectrum was recorded between 300 nm and 400 nm. In the presence of 1 mM EGTA, the calcium-free sample revealed an emission maximum at 356 nm. $CaCl_2$ was added to a final concentration of 2 mM, which shifted the emission maximum to 335 nm and increased the overall fluorescence yield by 30%. NEFA contains two Trp residues which flank the EF-hand pair, Trp233 preceding the first EF-hand site (a.p. 245-273) and Trp333 subsequent to the second EF-hand site (a.p. 297-325). 9 Tyr residues are present in NEFA, 3 of them in the C-terminal half of the protein, of which Tyr278 is placed in the spacer region between the two EF-hand sites. It is supposed that the conformation changes in the environment of Tyr278 reflect binding of Ca^{2+} to the paired EF-hand domain of NEFA.

To determine the equilibrium dissociation constants Kd_1 and Kd_2 of both calcium binding sites, the relative fluorescence of rcNEFA at 335 nm (ΔF335) was recorded at free Ca^{2+} concentrations ranging from 10 nM to 2×10^5 nM. The best fit titration curves derived from 15 titration experiments indicate the presence of one high (0.08 μM) and one low affinity (0.2 μM) calcium binding site in the protein (Fig. 3). The change of fluorescence at 335 nm could be reversed upon addition of excess EGTA (4 mM) to the Ca^{2+} titrated NEFA solution. The mutant proteins ΔEF1+2 and ΔEF1 lacking Tyr278 located between the two EF-hand motifs did not show any Ca^{2+} dependent change at ΔF335 (data not shown).

Circular dichroism (CD). A calcium-induced conformation change of rcNEFA was detected by circular dichroism spectroscopy. Far ultraviolet spectra were recorded over 190-240 nm (Fig. 4). Analyses of the CD spectra in terms of known secondary structure elements [30] suggest that the change occurs in an α-helical region of rcNEFA. The α-helix content increases from 30% to 43% in the presence of 100 μM Ca^{2+}, but not in the presence of 100 μM Mg^{2+} ions (data not shown), without significant change in other types of secondary structures (Table 3). ΔEF1+2 did not show any significant change of α-helix content under the same conditions (Fig. 4B and Table 3). These results indicate that the conformational change of NEFA in the presence of Ca^{2+} is mediated by the EF-hand sites. For the mutant ΔEF1, CD spectra could not be recorded due to the low level of recombinant protein expression.

Limited proteolysis. Another approach to elucidate the observed calcium-dependent structural change was the partial tryptic digestion of rcNEFA, ΔEF1+2 and calmodulin. The tryptic digestion patterns (Fig. 5A) display differences in the presence of Ca^{2+} ions in the case of rcNEFA and calmodulin, while this was not observed for ΔEF1+2. The degradation of calmodulin in the presence of Ca^{2+} is strongly reduced, whereas this effect is limited but visible in the case of rcNEFA. Investigating the effect of Mg^{2+} ions, it could be demonstrated for rcNEFA that Mg^{2+} in the same concentration did not evoke the described calcium dependent effects (Fig. 5B).

DISCUSSION

Heterologous overexpression of proteins in *P. pastoris* is inexpensive and straightforward as well as free of

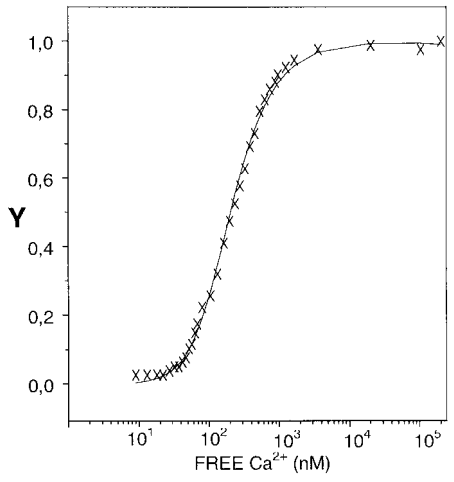

FIG. 3. Calcium binding to rcNEFA observed by fluorescence titration. Y: the total change in fluorescence emission at 335 nm is plotted as function of the free Ca^{2+} ion concentration. Free metal concentration in solution was controlled by means of an EGTA containing buffer and was calculated using the program "*Calcium*." The crosses represent the result from the binding assay. The solid line denotes the best fit function Y_{fit} of the data using the procedure described in the text.

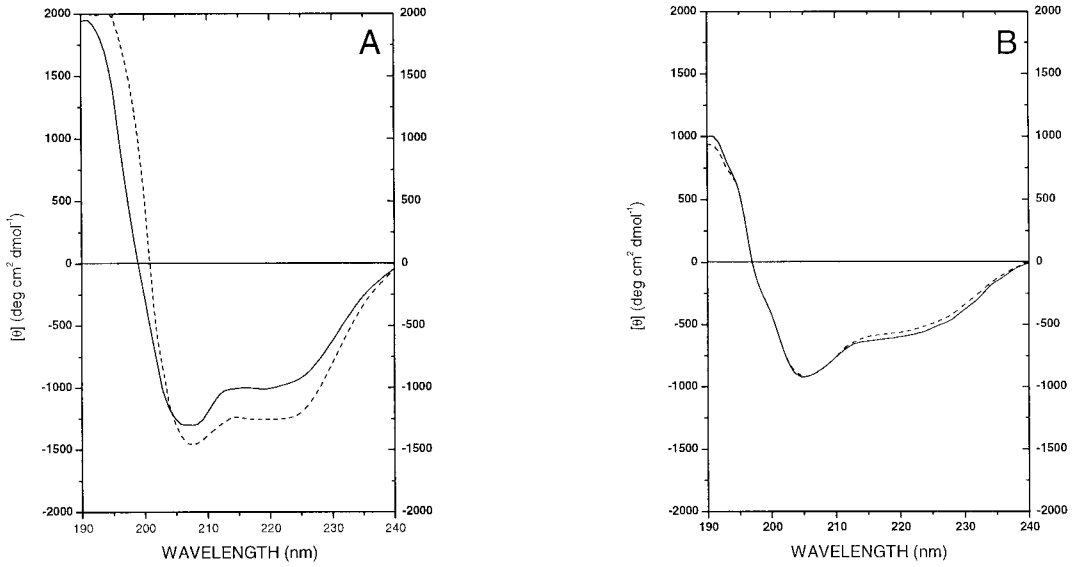

FIG. 4. Circular dichroism spectra of rcNEFA (A) and ΔEF1+2 (B) in the presence and absence of Ca^{2+}. Far-UV scans were recorded at a protein concentration of 0.1 μg/μl in 0.01 M sodium phosphate buffer, pH 7.2 at 25°C in a resolution of 10 mdeg in absence of Ca^{2+} (1 mM EGTA), represented by the solid line (—) and in the presence of 200 μM $CaCl_2$, shown by the broken line (- - -). The spectra were fitted by combination of reference spectra with the parameters given in Table 3.

biohazards. Therefore it is suitable to produce large amounts of protein for biochemical experiments. Mouse epidermal growth factor [14], bovine lysozyme [31] and human aspartic proteinase cathepsine E [32] were expressed at high levels in *P. pastoris*. Recombinant NEFA and its mutants were also expressed successfully and secreted into the culture medium, however, 10-50% were found to be proteolytically degraded. This proteolytic degradation was not prevented by induction at lower temperatures of 20-25°C or at a pH below 6.0 in the culture medium. Increasing the pH to 7.0-7.5 caused the inhibition of recombinant NEFA expression (data not shown).

To separate the full length proteins from the degraded fragments we chose gel chromatography in 6 M urea and subsequent rechromatography by RP-HPLC.

TABLE 3

Secondary Structure of Calcium-Saturated (+ Ca^{2+}) and Calcium-Depleted (− Ca^{2+}) rcNEFA and ΔEF1+2

Secondary structure	Fraction + Ca^{2+} rcNEFA	Fraction − Ca^{2+} rcNEFA	Fraction + Ca^{2+} ΔEF1+2	Fraction − Ca^{2+} ΔEF1+2
α-helix	43%	30%	19%	19%
β-sheet	43%	38%	53%	53%
Remainder	13%	31%	27%	28%
Total	99%	99%	99%	100%

Note. Values were derived from the circular dichroic spectra (Fig. 4A and 4B) according to the method of Provencher and Glöckner [30]. The samples were measured in a volume of 250 μl containing 0.1 μg/μl protein solution in 0.01 M sodium phosphate buffer, pH 7.2 at 25°C.

These methods were carried out to supply sufficient quantities of purified protein for calcium binding assays. Alternatively, ion exchange chromatography on Bio-scale DEAE (Bio-Rad) and MONOQ (Pharmacia) under various conditions of pH-range and salt concentration were tried to separate the full length protein from the degraded fragments. This approach was less efficient, probably because of shared properties among the different fragments and the full length proteins. Comparing batches of the purified proteins obtained by non-denaturing procedures and the proteins renaturated from denaturing purification did not reveal any detectable difference in CD and in protein fluorescence applications. The N-termini of the recombinant NEFA variants were not homogeneous, probably caused by unspecific KEX2 endopeptidase cleavage or other unknown endopeptidase activity in the secretory pathway.

The initial purpose for adding a Cys residue to the N-terminus of the rcNEFA variant was to get the prerequisite for labeling of the protein with biotin or fluorescein. However, the introduced Cys was only present in the minor population of rcNEFA and therefore this construct was not appropriate for labeling. The rNEFAc protein containing the introduced Cys at the C-terminus could be isolated as a full length protein containing Cys and was successfully used for labeling and immobilization experiments.

To distinguish between the two EF-hand calcium-binding properties and to analyze the function of Ca^{2+}-coordinated amino acid residues we generated, based on known features of the EF-hand motifs [33, 34], two

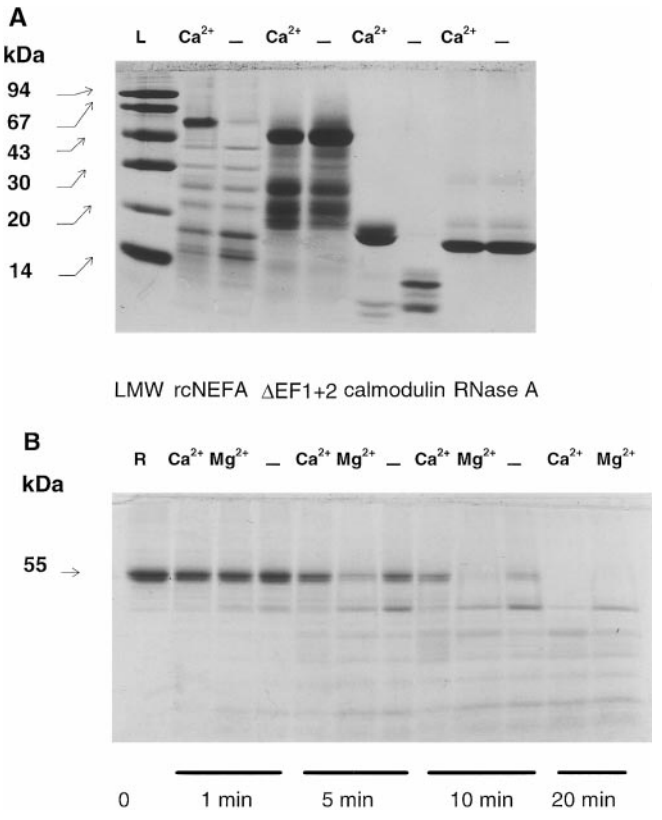

FIG. 5. Limited tryptic digestion experiments. (A) Effect of Ca^{2+} ions on the tryptic digestion of rcNEFA, ΔEF1+2, calmodulin and RNase A. 20 μg of rcNEFA, ΔEF1+2, calmodulin, and RNase A were digested in a volume of 80 μl 0.1 M Tris/HCl, pH 8.5 with trypsin (Boehringer-Mannheim) at a ratio of 1:1000 (w/w) enzyme to protein in the presence of 1 mM EGTA or 100 μM $CaCl_2$ at 20°C. The reaction was stopped after 10 min by boiling 10 μl aliquots in Laemmli-sample buffer. The fragmentation was analyzed by applying 10 μl samples analyzed without mercaptoethanol on a 15% SDS–PAGE. The gel was stained with Coomassie brilliant blue R-250. Lane L contains the LMW marker proteins. (B) Kinetics of tryptic cleavage of rcNEFA in the presence of 100 μM $CaCl_2$, 100 μM $MgCl_2$ and 1 mM EGTA (—). 10 μg rcNEFA was digested with trypsin at 20°C for the indicated time in a volume of 80 μl 0.1 M Tris/HCl, pH 8.5. The reaction was stopped as described before (see A). 10 μl of the samples were analyzed without mercaptoethanol on a 15% SDS–PAGE. The gel was stained with Coomassie brilliant blue R-250. Lane R contains uncleaved rcNEFA, without trypsin, after 30 min at RT in 0.1 M Tris/HCl, pH 8.5.

internal deletion mutants (Fig. 1) and several amino acid exchange mutants in the EF-hand domain (data not shown). For quantitative determination of Ca^{2+}-binding to recombinant NEFA variants, the fluorescent calcium indicator FURA-2 was used to avoid handling radioactive calcium isotopes. By this assay the free Ca^{2+} ions in the reaction mixture were measured. In spite of the critical filtration step to recover the unbound Ca^{2+} ions from the reaction mixture, we obtained highly reproducible values. The results suggest one mol of NEFA binds 2 mol of Ca^{2+} ions. The calcium-dependent fluorescence titration of the protein indicates the presence of one high- and one low-affinity calcium-binding site for NEFA. The deletion of the first EF-hand site in the ΔEF1 variant caused a dramatic increase in Kd and abolished any detectable calcium binding. As far as known, EF-hand Ca^{2+} binding proteins in general possess paired EF-hand domain structures as essential elements for the internal organization of the two cooperating EF-hand sites in different conformation stages [28, 35] as well as for the functional properties of each individual Ca^{2+}-binding site [36, 37].

As shown by limited proteolysis of recombinant NEFA, Ca^{2+} ions stabilize the protein against trypsin. This structural reorganization of the molecule upon Ca^{2+} binding may result in a more globular formation yielding a reduced proteolytic susceptibility, as described for a Troponin-C component [24]. In contrast, ΔEF1+2 appears equally prone to tryptic digestion under conditions where NEFA is being nearly completely digested indicating the presence of exposed cleavage sites in the deleted region. The expressed ΔEF1 mutant protein in *P. pastoris* was accompanied by rapid intracellular degradation implying that Ca^{2+} binding to the paired EF-hand domain is important for correct folding of the entire molecule, as described above. A variant lacking the second EF-hand site and various amino acid exchange mutants of the Ca^{2+}-coordinated amino acid residues in the EF-hand domain are currently in preparation for detailed analyses of the Ca^{2+} binding conditions and the effects on the Ca^{2+} dependent conformation states.

The successful heterologous expression of the human NEFA protein and the mutants enables further studies on crystallization of the protein and on protein/protein interactions.

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