Human rhinovirus 2A proteinase mutant and its second-site revertants

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The 2A proteinases of human rhinoviruses are cysteine proteinases with marked similarities to serine proteinases. In the absence of a three-dimensional structure, we developed a genetical screening system for proteolytic activity and identified Phe-130 as a key residue. The mutation Phe-130 → Tyr almost completely inhibited enzyme activity at 37 °C; activity was, however, partially restored by the following exchanges: Ser-27 → Pro, His-135 → Arg or His-137 → Arg. To investigate this phenotypic reversion, 2A proteinases with the mutations Phe-130 → Tyr, Phe-130 → Tyr/His-135 → Arg, Phe-130 → Tyr/His-137 → Arg, His-135 → Arg or His-137 → Arg were expressed in *Escherichia coli* and purified. None of these mutations affected the affinity of the enzyme for a peptide substrate. However, the temperature-

dependence of enzyme activity, as assayed by cleavage of a peptide substrate and by monitoring the toxicity of the proteinases towards the *E. coli* strain BL21(DE3), and the structural stability, as monitored by 8-anilino-1-naphthalenesulphonic acid fluorescence and CD spectrometry, were affected. The thermal transition temperatures for both the activity and the stability of the Phe-130 → Tyr 2A proteinase were reduced by about 17 °C compared with the wild-type enzyme. The presence of the additional mutations His-135 → Arg or His-137 → Arg in the Phe-130 → Tyr mutant increased temperature stability by 3 °C and 6 °C respectively. Thus essential interactions exist within the C-terminal domain of human rhinoviral 2A proteinases which contribute to the overall stability and integrity of the enzyme.

INTRODUCTION

Human rhinoviruses (HRVs) are members of the picornavirus family. The RNA genome contains a single open reading frame encoding a polyprotein of about 2100 amino acids [1] (see Figure 1). This polyprotein is subsequently cleaved in the nascent state by virally encoded proteinases [2–4]. In rhinoviruses, as in the related polio- and coxsackie-viruses, the first proteolytic event is carried out by the viral proteinase 2A, which cleaves intramolecularly at its own N-terminus, separating the capsid from the non-capsid proteins [5,6]. All but one of the remaining cleavages of the polyprotein are carried out by a second virus-encoded proteinase 3C or its precursor 3CD [7,8]. The one remaining cleavage, between VP4 and VP2, has been proposed to occur autocatalytically [9].

From sequence comparisons the 2A proteinases, like the 3C proteinases, show a strong relationship to serine proteinases [6,10–13]. Recently the three-dimensional structure of two 3C proteinases, from Hepatitis A virus and HRV14, have been solved [14,15].

Despite their obvious similarities to the 3C proteinases, the 2A proteinases show markedly different properties in the determinants of substrate specificity; in addition, they contain one tightly bound zinc ion, probably co-ordinated by Cys-52, Cys-54, Cys-112 and His-114 [16]. The zinc ion has been shown recently to be required for structural integrity [17]. From site-directed mutagenesis experiments and sequence comparisons, the residue Cys-106 has been postulated to be the active-site residue in HRV2 2A proteinase, with His-18 and Asp-35 acting as the other members of the catalytic triad [6]. However, as a three-dimensional structure is not yet available, information on structure–function relationships for the 2A proteinases can only be obtained from genetical and biochemical studies. To this end, we developed an *in vivo* colorimetric system (called proteinase trapping) using α -complementation to screen for proteolytic activity. In this system, the cDNA coding for the C-terminus of VP1 of HRV2 and the entire 2A proteinase was fused to the N-terminus of the *LacZ* α -fragment of M13mp18 [18]. On expression, an active proteinase cleaved itself from the α -fragment, allowing the production of blue plaques; inactive proteinases gave rise to a C-terminal extension of the α -fragment, thus hampering α -complementation and leading to white plaques. Analysis of inactive mutants obtained showed that mutations at amino acid Phe-130 were particularly informative; in the *in vivo* assay, exchanging this phenylalanine for serine or leucine led to complete inactivation, whereas a barely detectable level of activity was observed when Phe-130 was replaced by tyrosine (Table 1). Indeed, the level of activity of the Phe-130 → Tyr mutant was below that detectable in an *in vitro* assay [18]. The screening system was then employed to obtain revertants with second-site mutations which re-activated the Phe-130 mutants. Only mutants containing Phe-130 → Tyr could be re-activated; any one of the

Figure 1 Schematic representation of the HRV RNA genome

Coding regions are shown as open boxes, and non-coding regions as lines. The virally encoded protein VPg is covalently linked to the 5' end of the RNA. The capsid proteins are VP1–VP4; the remainder are non-structural. UTR, untranslated region.

Abbreviations used: HRV, human rhinovirus; ANS, 8-anilino-1-naphthalenesulphonic acid; DTT, dithiothreitol; IPTG, isopropyl β -D-thiogalactoside.

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Table 1 Colour of plaques and *cis* cleavage activity of 2A proteinase Phe-130 mutants and revertants using the M13 screening system

The results are compared with those for wild-type 2A proteinase. Data taken from [19].

2A proteinase	Plaque colour	Cleavage activity (<i>cis</i>)
Wild-type	Dark blue	+++
Phe-130 → Tyr	Light blue	—
Phe-130 → Ser	White	—
Phe-130 → Leu	White	—
Phe-130 → Tyr/Ser-27 → Pro	Blue	++
Phe-130 → Tyr/His-135 → Arg	Blue	++
Phe-130 → Tyr/His-137 → Arg	Blue	+++

mutations Ser-27 → Pro, His-135 → Arg or His-137 → Arg restored enzyme activity, albeit not to the wild-type level [19].

The present paper describes the characterization of the HRV2 2A proteinase mutant Phe-130 → Tyr and of the two second-site revertants Phe-130 → Tyr/His-135 → Arg and Phe-130 → Tyr/His-137 → Arg.

MATERIALS AND METHODS

Materials and general methods

Restriction enzymes and DNA-modifying enzymes were from Boehringer Mannheim or New England Biolabs (Beverly, MA, U.S.A.), and *Taq* (*Thermus aquaticus*) DNA polymerase was from Promega (Madison, WI, U.S.A.). Manipulation and preparation of DNA was by standard protocols [20]. DNA was sequenced as described [21] by using T7 DNA polymerase from Pharmacia and [α -³²P]dATP from Amersham, or an automated laser fluorescence sequencer (A. L. F.) and an Autoread Sequencing kit from Pharmacia. Oligonucleotide and oligopeptide synthesis was performed as described [6,22].

Bacterial strains and vectors

pET expression plasmids [23] were maintained in the *Escherichia coli* K12 strain HMS174 [*recA1 hsdR*(r_{K12}⁺,m_{K12}⁺) rif^r F⁺] and expressed in the pLysE or pLysS derivatives of *E. coli* B strain BL21(DE3) [*hsdS*(r_B⁺,m_B⁺) *gal ompT* F⁺(λ Its857 *ind1 Sam7 nin5 lacUV5-T7* gene 1)]. The LB medium contained ampicillin (100 μ g/ml) and chloramphenicol (30 μ g/ml) as required. To examine the thermal stability of the 2A proteinase mutants in bacteria, the plasmid DNAs were transformed into *E. coli* BL21(DE3) cells made competent by treatment with 50 mM CaCl₂ [20]. Transformants were then selected at the indicated temperature on agar plates containing ampicillin at 100 μ g/ml.

Vector M13/2A

This M13 vector has been described [18], and contains nucleotides 3075–3586 of HRV2 [24] fused in-frame with codon 45 of the β -galactosidase gene. Two stop codons are present after the HRV2 cDNA sequences, so that induction of β -galactosidase expression with isopropyl β -D-thiogalactoside (IPTG) leads to the production of a fusion protein containing the 45 N-terminal amino acids of β -galactosidase, the last 28 amino acids of VP1 and all of the 2A proteinase. Derivatives of M13/2A bearing the mutations Phe-130 → Tyr, Phe-130 → Tyr/His-135 → Arg and Phe-130 → Tyr/His-137 → Arg of the 2A proteinase were obtained previously [19]. To obtain the single mutants His-135 → Arg and His-137 → Arg, site-directed mutagenesis using PCR

was carried out on M13/2A. Phage DNAs containing the desired mutations were checked by sequencing the entire 2A proteinase coding region.

Plasmid pET8c/ATG-2A

The expression vector pET8c/ATG-2A (see Figure 2) contains HRV2 cDNA nucleotides 3161–3586, which code for all 142 amino acids of HRV2 2A, preceded by an ATG initiation codon; two stop codons are present at the end of the 2A coding sequence followed by a *Hind*III site. Expression plasmids containing the HRV2 2A gene fragments bearing the single mutations Phe-130 → Tyr, His-135 → Arg or His-137 → Arg, or the double mutations Phe-130 → Tyr/His-135 → Arg or Phe-130 → Tyr/His-137 → Arg, were created by exchanging the 0.4 kb *Bst*EII/*Hind*III fragment of pET8c/ATG-2A with the *Bst*EII/*Hind*III fragments of the corresponding M13/2A mutants (Figure 2). Similarly, an expression plasmid for HRV2 2A proteinase lacking amino acids Gly-104, Gly-107 and Gly-108 (designated 2A proteinase Δ 3Gly) was generated from M13/2A Δ 3Gly [18].

Expression of the 2A proteinase mutants

For expression, a 500 ml stationary-phase culture of *E. coli* BL21(DE3)pLysE bearing the appropriate pET8c/ATG-2A derivative was diluted 1:9 (v/v) in 4 litres of fresh LB medium containing ampicillin and chloramphenicol. The bacterial culture was incubated for about 3 h with shaking at 150 rev./min until an A_{595} of 0.5–0.6 was reached. Incubation of bacteria bearing the pET8c/ATG-2A derivatives with the single mutations His-135 → Arg or His-137 → Arg was carried out at 30 °C. After addition of IPTG to a final concentration of 0.2 mM, the cultures were incubated for a further 6 h at 30 °C. For the 2A mutants Phe-130 → Tyr, Phe-130 → Tyr/His-135 → Arg and Phe-130 → Tyr/His-137 → Arg, which appeared to be thermally unstable, the procedure was identical except that incubation was performed at 15–18 °C and IPTG was added to a final concentration of 0.1 mM. The bacteria were harvested by centrifugation at 6000 *g*, rinsed once with 100 ml of washing buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM EDTA and 5 mM dithiothreitol (DTT), and the cell pellets stored at –80 °C.

Purification of the recombinant 2A proteinase mutants

In general, purification was carried out as described [25,26], but with the following modifications. After ammonium sulphate precipitation of the cleared cell lysate, proteins were dissolved in 24 ml of buffer A (50 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 5 mM DTT, 5% glycerol) and desalted on Sephadex G-25 at 4 °C before application on to a Pharmacia FPLC HR10/10 Mono-Q column. Chromatography was carried out at 4 °C, using a gradient of 190 ml of 0–40% buffer B (buffer A containing 1 M NaCl) and 20 ml of 40–100% buffer B. The HRV2 2A proteinase mutants, like the wild-type 2A proteinase [25], eluted at around 300–400 mM NaCl. Fractions containing proteinase were identified by SDS/PAGE and staining with Coomassie Brilliant Blue R250, pooled and applied directly to a Pharmacia Superdex 75 Hiload 26/60 column pre-equilibrated with 3 bed volumes of buffer A and water-cooled to 4 °C. Proteins were then eluted with buffer A. HRV2 2A proteinase mutants, like the wild-type proteinase, eluted with an apparent molecular mass of 30 kDa. Fractions from the A_{280} peak were examined by SDS/PAGE and Coomassie staining, and those containing 2A proteinase at an estimated purity of more than 98% were pooled. The pooled fractions were then concentrated about 30-fold in an Amicon Centriprep 10 cell to a final volume

of about 800 μ l; the preparations were as pure as wild-type preparations previously described [16,25].

The concentrations of the purified 2A proteinases were determined by measuring the absorbance at 280 nm in 0.1 M phosphate buffer, pH 7.1, using a molar absorption coefficient at 280 nm of 20000 M⁻¹·cm⁻¹ [25].

K_m determinations of the 2A proteinase mutants

The activity of the purified HRV2 2A proteinase mutants was measured as described [22,24] using the peptide P8–P8', corresponding to the eight C-terminal residues of VP1 and the eight N-terminal residues of wild-type 2A proteinase. After a pre-incubation for 30 min at 15 °C, 2.5 μ g of purified 2A proteinase and 66, 132, 198, 264, 330, 397, 462 or 529 μ mol of peptide P8–P8' were incubated at 15 °C in a final volume of 265 μ l. Aliquots of 50 μ l were removed after 20, 40, 60, 80, 100 and 120 s, mixed with 200 μ l of cold 0.5 M perchloric acid and spun through an ULTRAFREE tube (10000 NMWL; Millipore) for 30 min at 4 °C. The samples were then analysed by reverse-phase HPLC on a Merck Supersphere C18 column as described [6]; elution was performed using 0.1 % trifluoroacetate and a gradient of 10–40 % acetonitrile [22]. Calculation of initial rates and derivation of K_m values from Lineweaver–Burk diagrams was as described [22].

Temperature-dependence of the proteolytic activity of the 2A proteinase mutants

To determine the influence of temperature on the cleavage activity of the 2A proteinase mutants, the peptide P8–P8' was again used as substrate. Peptide and proteinase were preincubated separately for 30 min at temperatures between 10 and 45 °C in steps of 2.5 °C. After a further preincubation for 30 min at the assay temperature of 22.5 °C, 212 μ mol of peptide (32.5 μ l of a 8 mg/ml solution) and 6.5 μ g of 2A proteinase mutant (650 μ l of a 10 μ g/ml solution) were mixed and incubated at 22.5 °C. Aliquots of 50 μ l were removed every 20 s from 20 to 240 s, and after 15 min. The samples were then processed as described above. The initial velocity (v) at each temperature assayed was determined graphically. For comparison purposes, the measured velocities for each temperature value were normalized to the highest value obtained in each data set.

CD spectroscopy

The thermal stability of the wild-type and mutant 2A species was monitored by the ellipticity changes at 216 nm using a JASCO 600 CD spectrometer as described [17]. The data are expressed as mean residual ellipticity (molecular mass of 16209 Da; 142 amino acid residues). The spectra were recorded using 100 μ g/ml protein in 10 mM phosphate buffer, pH 7.5.

Fluorescence spectroscopy

Fluorescence spectra were measured with a Hitachi F-2000 fluorescence spectrometer. Thermal unfolding was carried out as described [17]. Excitation was at 390 nm, with fluorescence being recorded at 488 nm.

RESULTS AND DISCUSSION

Expression and purification of recombinant HRV2 2A proteinase mutants

In order to produce the amounts of pure HRV2 2A proteinase mutants required, it was necessary to modify the expression and

Figure 2 Construction of five pET8c/ATG-2A derivatives bearing the single mutations Phe-130 $\rightarrow$ Tyr, His-135 $\rightarrow$ Arg and His-137 $\rightarrow$ Arg and the double mutations Phe-130 $\rightarrow$ Tyr/His-135 $\rightarrow$ Arg and Phe-130 $\rightarrow$ Tyr/His-137 $\rightarrow$ Arg

(A) Within the gene for tetracycline resistance (tet^r), the expression plasmid pET8c/ATG-2A contains the ϕ 10-promotor (ϕ 10) and ϕ -terminator (T ϕ) of the bacteriophage T7. Between these two elements lies a gene fragment coding for the 142 amino acids of HRV2 2A proteinase, which is preceded by a methionine codon. SD depicts the Shine–Dalgarno sequence. (B) The single BstEII and HindIII sites, at amino acid position 10 and the C-terminus of the 2A proteinase respectively, were used to exchange the enclosed sequence for the corresponding sequences of M13/2A constructions bearing the mutations Phe-130 $\rightarrow$ Tyr (1), His-135 $\rightarrow$ Arg (2), His-137 $\rightarrow$ Arg (3), Phe-130 $\rightarrow$ Tyr/His-135 $\rightarrow$ Arg (4) and Phe-130 $\rightarrow$ Tyr/His-137 $\rightarrow$ Arg (5).

purification system which was described for the wild-type proteinase [25]. In this system, the wild-type proteinase is expressed as a fusion protein with 28 amino acids of VP1; the 2A proteinase cleaves itself from the growing polypeptide between the C-terminus of VP1 and its own N-terminus, resulting in the accumulation of mature 2A proteinase. The decrease in the *cis* activity of the Phe-130 $\rightarrow$ Tyr mutant precluded the use of this system, as mostly uncleaved fusion protein could be expected. Thus the expression vector pET8c/ATG-2A (Figure 2A), which produces the mature 2A proteinase with an extra methionine residue at the N-terminus, was employed. The single cleavage sites for BstEII and HindIII allowed the wild-type 2A cDNA fragment to be exchanged for those from M13/2A DNAs containing the single mutations Phe-130 $\rightarrow$ Tyr, His-135 $\rightarrow$ Arg and His-137 $\rightarrow$ Arg, and the double mutations Phe-130 $\rightarrow$ Tyr/His-135 $\rightarrow$ Arg and Phe-130 $\rightarrow$ Tyr/His-137 $\rightarrow$ Arg (Figure

Table 2 Kinetic constants of the wild-type and mutant 2A proteinases

Individual values are given, with values in parentheses representing averages. The $V_{\max}$ values assume that all protein samples were 100% active.

2A proteinase	$10^4 \times K_m$ (mol/litre)	$10^5 \times V_{\max}$ (mol/min per litre)
Wild-type	5.4, 5.3, 5.5 (5.4)*	6.7, 5.7, 7.1 (6.5)
Phe-130 → Tyr	4.3, 5.3 (4.8)	6.6, 7.0 (6.8)
His-135 → Arg	5.0, 7.4 (6.2)	5.4, 8.1 (6.7)
His-137 → Arg	2.4, 3.0 (2.7)	4.3, 4.3 (4.3)
Phe-130 → Tyr/His-135 → Arg	8.9, 6.1 (7.5)	17.5, 15.9 (16.7)
Phe-130 → Tyr/His-137 → Arg	4.3, 6.7 (5.5)	6.9, 7.6 (7.2)

* Value from [22].

2B). For expression, the plasmids were transformed into the pLysE derivative of *E. coli* strain BL21(DE3).

After optimizing temperature, time of incubation and IPTG concentration, the expression of the 2A proteinase mutants was carried out as described in the Materials and methods section; all but the Phe-130 → Tyr mutants were expressed at 30 °C. As no functional protein could be recovered at 30 °C with the single Phe-130 → Tyr mutant, expression of mutants bearing this exchange was carried out between 15 and 18 °C.

The recombinant 2A proteinases were then purified as described in the Materials and methods section. Once again, the apparent temperature sensitivity of the Phe-130 → Tyr 2A proteinase mutants required that all purification steps be carried out on ice or in the cold-room. Yields of recombinant HRV2 2A proteinase mutants were about 7–16 mg from 4 litres of *E. coli* culture. The purity of the proteinase preparations was greater than 98% as judged by SDS/PAGE (results not shown).

Determination of the K_m values of the 2A proteinase mutants

To investigate the affinity of the mutant 2A proteinases for a peptide substrate, the K_m values were determined. Enzyme kinetics were carried out as described in the Materials and methods section, using peptide P8–P8' as substrate. Two independent determinations of the Michaelis–Menten parameters were carried out for each 2A proteinase mutant. For the K_m values, a Lineweaver–Burk diagram was constructed from the mean values of the two determinations (Table 2). The K_m of the wild-type HRV2 2A proteinase ($K_m = 5.4 \times 10^{-4}$ mol/l), obtained from three determinations using different 2A preparations [22], served as a control. The K_m values of the Phe-130 → Tyr ($K_m = 4.8 \times 10^{-4}$ mol/l), His-135 → Arg ($K_m = 6.2 \times 10^{-4}$ mol/l) and Phe-130 → Tyr/His-137 → Arg ($K_m = 5.5 \times 10^{-4}$ mol/l) mutants showed no significant difference from that of the wild-type. Although the Phe-130 → Tyr/His-135 → Arg mutant ($K_m = 7.5 \times 10^{-4}$ mol/l) exhibited a slightly lower and the His-137 → Arg mutant ($K_m = 2.7 \times 10^{-4}$ mol/l) a slightly higher substrate affinity, the differences in the properties of the mutants cannot be accounted for by changes in substrate affinity. Similarly, no significant differences in the $V_{\max}$ values were observed (Table 2).

Performance of a temperature sensitivity assay

As an apparent temperature sensitivity of the Phe-130 → Tyr mutant had been identified during proteinase expression and purification, the effect of temperature on the proteolytic activity of each 2A proteinase mutant was determined (Figure 3). Proteinase and substrate peptide P8–P8' were preincubated

Figure 3 Effect of preincubation temperature on the proteolytic activity of the 2A proteinase mutants

Preincubation and assay of activity were as described in the Materials and methods section. Proteolytic activity is presented as a percentage of maximal activity for each mutant (taken as 100%). Symbols: □, wild-type; ○, Phe-130 → Tyr; ■, His-135 → Arg; ●, His-137 → Arg; ▲, Phe-130 → Tyr/His-135 → Arg; △, Phe-130 → Tyr/His-137 → Arg.

separately for 30 min at different temperatures ranging from 10 °C to 45 °C. Proteinase and substrate were then united and residual proteinase activity was measured at 22.5 °C. Samples were taken every 20 s up to 240 s. The initial velocity at the indicated temperatures provides an insight into the influence of temperature on enzyme stability. Proteinase activity abolished by incubation at higher temperatures could not be restored by incubation at the assay temperature of 22.5 °C. Thus the Phe-130 → Tyr proteinase is indeed a thermally labile mutant; its enzyme activity was reduced by 50% by incubation at 28 °C for 30 min and completely abolished by incubation at 32.5 °C. In contrast, the wild-type enzyme and the single mutants His-135 → Arg and His-137 → Arg still retained 50% of their proteolytic activity after an incubation at 45 °C for 30 min. The double mutants Phe-130 → Tyr/His-135 → Arg and Phe-130 → Tyr/His-137 → Arg were also thermally unstable when compared with the wild-type proteinase. However, in comparison with the original mutant Phe-130 → Tyr, the temperatures required for 50% and 100% inactivation were 3 °C higher for the Phe-130 → Tyr/His-135 → Arg mutant and 6 °C higher for the Phe-130 → Tyr/His-137 → Arg mutant.

Investigation of the structural stability of the mutants by CD spectroscopy and 8-anilino-1-naphthalenesulphonic acid (ANS) fluorescence

The thermal stability of wild-type and mutant forms of HRV2 2A proteinase was further monitored by CD spectroscopy (Figure 4A). The thermal transition of wild-type 2A was earlier shown to occur around 53 °C [17]. All mutants exhibited significantly lower transition points (summarized in Table 3). The mutant Phe-130 → Tyr turned out to be structurally the least stable and Phe-130 → Tyr/His-137 → Arg the most stable mutant. These data correlate well with the temperature sensitivity observed in the enzyme kinetics experiments which demonstrated the same order of thermostability, although the transition temperatures

Figure 4 Thermal stability of wild-type and mutant 2A proteinases from HRV2

(A) Thermal unfolding monitored by CD spectroscopy. Data are expressed as mean residual ellipticity (θ) (degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$) at 216 nm. (B) Temperature-dependence of ANS (50 μ M) fluorescence in the presence of 50 μ g/ml native or mutant 2A proteinase.

Table 3 Thermal transition temperatures of wild-type and mutant 2A proteinases

Transition points were determined by enzyme kinetics (50% residual activity, determined from Figure 3), CD spectroscopy and ANS fluorescence spectroscopy.

2A proteinase	Transition temperature (°C)		
	Kinetics	CD	ANS
Wild-type	45.0	53.0	53.4
Phe-130 $\rightarrow$ Tyr	28.0	37.6	32.4
Phe-130 $\rightarrow$ Tyr/His-135 $\rightarrow$ Arg	31.0	39.7	34.2
Phe-130 $\rightarrow$ Tyr/His-137 $\rightarrow$ Arg	34.0	42.8	35.6

determined by CD spectroscopy are higher. This reflects the fact that overall secondary structure elements which are monitored by CD spectroscopy are more stable than the folding required for enzymic activity.

This was further verified by fluorescence spectroscopy (Figure 4B). Here, the thermal transition is monitored by the binding of ANS to the denaturing molecule. This binding is transient, since thermal denaturation generates a molten globule-like structural intermediate which unfolds fully at higher temperatures [17]. The transition temperatures determined by this technique were generally lower than those from CD measurements, although they show the same order of thermal sensitivity as determined by the

other methods (Table 3). These temperatures are again higher than those from kinetics experiments, indicating the greater sensitivity of enzymic activity compared with structural changes. In this context, it is notable that all three mutations have an effect not only on the overall stability of the proteinase, as determined by the enzymic activity and fluorescence measurements, but also on the secondary structure elements. This is also reflected by the reduced CD intensities of the mutants (results not shown).

The investigations on the effect of temperature on enzymic activity, as measured by peptide cleavage, and on protein folding, as measured by CD spectroscopy and ANS fluorescence, show clearly that the presence of the Phe-130 $\rightarrow$ Tyr mutation in the HRV2 2A proteinase leads to thermal instability of the enzyme. All three methods of measurement indicate that the presence of the hydroxy group of tyrosine reduces the thermal stability, with thermal transition taking place at a temperature approx. 17 °C lower than for the wild-type. The presence of arginine in place of histidine at either position 135 or 137 in the protein alleviates the thermal lability of the Phe-130 $\rightarrow$ Tyr mutant, increasing the transition temperature by about 3 °C and 6 °C respectively, although the double mutants are still less thermally stable than the wild-type.

Thermal stability of the wild-type proteinase and its mutants *in vivo*

The experiments described above show clearly that the Phe-130 $\rightarrow$ Tyr mutant is thermally labile. In the M13 screening system, which is performed at 37 °C, this led, therefore, to the loss of the ability of the 2A proteinase mutant to cleave itself from the growing peptide chain and thus to an extension of the α -fragment of β -galactosidase. Such an α -fragment is impaired in its ability to undergo α -complementation and resulting plaques are white. Repeating the experiment at 30 °C should leave the Phe-130 $\rightarrow$ Tyr mutant active and reverse the phenotype. However, it is not possible to perform such an experiment, as the pili required for M13 infection are not expressed at temperatures below 34 °C [27].

In order to overcome this problem, we made use of the fact that the expression of viral proteinases is, under certain circumstances, toxic for the *E. coli* cell. Thus Baum et al. [28] showed that transformation of *E. coli* strain BL21(DE3) with a plasmid containing the HIV proteinase cDNA under the control of a T7 RNA polymerase promoter is only possible with the pLysS derivative. In this derivative, the episomal expression of T7 lysozyme inhibits any T7 RNA polymerase molecules generated before induction. In the absence of T7 lysozyme, the T7 RNA polymerase can transcribe the foreign gene; for the HIV proteinase, this level was sufficient to prevent the formation of stable transformants. This is also the case with the 2A proteinase of HRV2, as can be seen in Table 4. Using the bacterial strain BL21(DE3), essentially no transformants were obtained with a plasmid bearing the wild-type 2A proteinase; the generation of transformants was, however, normal in the pLysS derivative. In contrast, with a plasmid bearing the inactive 2A proteinase mutant Δ 3Gly, the number of colonies observed was independent of the strain used and the temperature of incubation. The results obtained with plasmids bearing the mutations Phe-130 $\rightarrow$ Tyr or Phe-130 $\rightarrow$ Tyr/His-135 $\rightarrow$ Arg were identical to those with the Δ 3Gly plasmid at 37 °C. However, at 34 °C both the number and size of the colonies with Phe-130 $\rightarrow$ Tyr/His-135 $\rightarrow$ Arg were reduced. Moreover, at 30 °C, no colonies were observed with either of the plasmids bearing the mutation Phe-130 $\rightarrow$ Tyr. These results suggest that the double mutant was still sufficiently active at 34 °C to influence the transformation of the bacteria,

Table 4 Transformation of *E. coli* BL21(DE3) strain with plasmid pET8c/ATG-2A bearing the indicated mutations

Strain...	Temperature (°C)...	Number of colonies			
		BL21(DE3)		BL21(DE3)pLysS	
		30	34	37	37
Wild-type		3	4	3	> 2000
Δ3Gly		> 2000	> 2000	> 2000	> 2000
Phe-130 → Tyr		3	> 2000	> 2000	> 2000
Phe-130 → Tyr/His-135 → Arg		4	> 1000*	> 2000	> 2000

* Indicates a smaller colony size.

whereas the single mutant was not. At 30 °C, however, both the original mutant and the revertant were active enough to prevent transformant formation. Therefore we conclude that the mutant 2A proteinases are also thermolabile in an *in vivo* situation.

The events behind the prevention of the generation of stable transformants in *E. coli* by the HRV2 2A proteinase or the HIV proteinase [28] remain obscure; as these proteinases have relatively strict requirements for substrate recognition, it seems likely that only a small number of proteins in the *E. coli* cell will be modified. Investigation of the proteins involved may shed light on events required for successful transformation of *E. coli* by plasmid DNA.

Given that residue Phe-130 and three of the surrounding six amino acids (Val-128, Asp-132, Arg-134) are conserved, it is likely that the mutation Phe-130 → Tyr will lead similarly to a thermally labile 2A proteinase in all rhino- and entero-viruses. It will thus be of interest to introduce this mutation into full-length viral cDNAs from which infectious RNA can be transcribed *in vitro*. Virus can then be generated by transfection of the RNA into HeLa cells and the properties of the viruses can be examined. Should the replication of these viruses be temperature-sensitive, this will allow temperature-shift experiments to be performed to examine in which stages of replication the viral proteinase is required. Experiments addressing this question are in progress.

In summary, a thermally unstable mutant and two revertants of the HRV2 2A proteinase have been expressed in bacteria and characterized biochemically. In the absence of crystallographic data for the HRV2 2A proteinase, this study presents important information with respect to intramolecular interactions and sites that are important for enzyme activity within the 2A proteinase.

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