Expression of the GTPase Activating Domain of the Neurofibromatosis Type 1 (NF1) Gene in Escherichia coli and Role of the Conserved Lysine Residue*

Lis A. Wissmuller and Alfred Wittinghofer
From the Max-Planck-Institut fur Medizinische Forschung, Abteilung Biophysik, Jahnstraße 29, 6900 Heidelberg, Germany

A small catalytic domain from the neurofibromatosis type 1 gene, NF1-333, consisting of 333 amino acids between residues 1197 and 1528, including an additional N-terminal methionine, was expressed in Escherichia coli as a soluble protein. Its catalytic activity under non-saturating conditions is similar to the full-length p120-GAP but different from truncated GAP-334. Under saturating conditions its kcat and Km are lower. Lys-1422, which is totally conserved in all GAP proteins, was mutated and the properties of the mutant protein investigated. Lys-1422 seems to be essential for the stability of the proteins and not for its catalytic activity.

Neurofibromatosis type 1 (NF1) is a very common autosomal dominant disorder in humans. Recently a gene was identified with exon-based mutations in patients suffering from NF1 neurofibromatosis (Cawthon et al., 1990; Wallace et al., 1991; Viskochil et al., 1990). Sequence analysis of the corresponding cDNA predicted a gene within which a region of approximately 400 amino acids had 30% similarity to the catalytic domain of yeast IRA proteins and the mammalian GTPase activating protein GAP (Xu et al., 1990a). These proteins accelerate the GTPase of wild-type p21 proteins, were mutated and the properties of the mutant protein investigated. Lys-1422 seems to be essential for the stability of the proteins and not for its catalytic activity.

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1 The abbreviations used are: NF1, neurofibromatosis type 1; GAP, GTPase activating protein; PCR, polymerase chain reaction; DTE, dithioerythritol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NF1-GRD, GAP-related domain of NF1.

2 For mutagenesis of the NF1-333 coding region, the EcoRI-HindIII fragment comprising 331 base pairs was cloned into M13 mp9. Mutations in the cDNA, 5'CAGTATTGGCTGATCGGTTTGA-3' (mutating base indicated with an asterisk). After amplification reaction to produce a fragment that codes for a 333-amino acid fragment and can be cloned into the NcoI-HindIII sites of the E. coli expression vector pTR9A (Ammann et al., 1988). Forward and reverse primer were 5'AGGGAGGCGCATGAAAAGACTATTGCTGATCGGTTTGA-3' and 5'CCTCCTCTTAAGCTTCTCTTAT'TACTCTGGAGAGGACCCTGATCGAAG-3', respectively.

3 The solution was chromatographed over a G-75 (Pharmacia) gel filtration column. Protein concentrations were determined following excess nucleotide was removed on a NAP-5 gel filtration column.
NF1 Expression

FIG. 1. Alignment of sequences containing a GTPase activating domain. The actual catalytic domain is black; additional homologies are shaded. The sequences are from man (H.s.), Saccharomyces cerevisiae (S.C.) and Schizosaccharomyces pombe (S.P.). The consensus sequence containing totally conserved residues (underlined) is from Wang et al. (1991).

Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific activity</th>
<th>$k_{cat}$</th>
<th>$K_M$</th>
<th>$T_{1/2}$</th>
<th>IC$_{50}$(NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF1-333</td>
<td>1575</td>
<td>1.4</td>
<td>0.3</td>
<td>70</td>
<td>200</td>
</tr>
<tr>
<td>NF1-333 (K1422M)</td>
<td>26–1443</td>
<td>?</td>
<td>?</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>p120-GAP $^d$</td>
<td>1833</td>
<td>19</td>
<td>9.7</td>
<td>ND</td>
<td>30–40$^c$</td>
</tr>
<tr>
<td>GAP-334$^d$</td>
<td>330</td>
<td>4.2</td>
<td>19</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ One unit is defined as the amount of GAP activity necessary to catalyze hydrolysis of 1 nmol of p21-bound GTP at 1 µM p21-GTP, in standard buffer, at 25 °C.

$^b$ $T_{1/2}$ is the temperature at which half the enzymatic activity is (irreversibly) lost during a 20-min incubation.

$^c$ IC$_{50}$(NaCl) is the concentration of NaCl necessary for 50% inhibition of GAP activity.

$^d$ These values are from Gideon et al. (1992).

$^e$ From McCormick et al. (1988) and Gibbs et al. (1988).

Fig. 3. Michaelis-Menten kinetics of the p21-GAP interaction. GTPase initial reaction rates were measured with a constant amount of NF1-333 and increasing concentrations of p21-GTP. By non-linear fitting we obtain the $K_M$ and $k_{cat}$ values shown in Table I.

Fig. 4. Thermal stability of wild-type and mutant K1422M NF1-333. Proteins in buffer are incubated at the indicated temperatures for 20 min and immediately analyzed for the ability to catalyze the GTPase reaction of p21-GTP. The data are plotted as the residual activity of the protein after incubation. , wild-type; , mutant.

Results and Discussion

Fig. 1 shows the alignment of the GTPase activating domain of various proteins (Imai et al., 1991) and the derived consensus sequence in the most highly conserved regions.
Amino acids totally conserved in all GAPs and GAP-like proteins are underlined (Wang et al., 1991). NF1 contains a region which is considered the actual catalytic domain and in addition a region of homology to the yeast IRA proteins, which is not present in p120-GAP. We have constructed an E. coli plasmid from which a supposedly minimal catalytic domain of NF1 comprising 333 amino acids can be expressed. It covers a similar region of amino acid sequence as GAP-334 described earlier (Halenbeck et al., 1990; Gideon et al., 1992). Pure NF1-333 can be obtained by a three-column purification procedure involving ion-exchange chromatography on Q-Sepharose, chromatofocusing on PBE 94, and gel filtration over G-75 as described in detail under “Materials and Methods.” Fig. 2 shows an SDS-polyacrylamide gel summarizing the purification steps. From 100 g of cells, 3.4 mg of purified NF1-333 can be obtained, which corresponds to 9% of the GAP activity originally present in the crude bacterial extract.

On a SDS-polyacrylamide gel electrophoresis gel NF1-333 has an apparent molecular mass of 34 kDa, whereas the calculated molecular mass is 38 kDa. Fig. 2 shows that in some NF1-333 preparations (lane 5) the protein runs as a double band containing an additional 33-kDa impurity, and it appears that sometimes the lower band is purified. This seems to be a proteolysis product obtained during the preparation. The double band product has been sequenced and has been found to contain a uniform N terminus with the sequence METVLADRFERLVE, as expected from the construction of the plasmid. This means that the second band is a breakdown product of NF1-333 and has been digested at the C terminus, even though protease inhibitors have always been used.

Table I lists the enzymatic properties of the purified protein. When the specific activity is measured under conditions where it is linearly dependent on the concentration of NF1-333 (5 nM) and p21 (1 μM p21-GTP), it is found to be approximately 3 times smaller than the specific activity of full-length human GAP on a molar basis (the molecular masses are 38 kDa compared with 120 kDa) and much higher than the specific activity of the C-terminal fragment GAP-334, which has a comparable molecular mass (Table I) (Halenbeck et al., 1990; Gideon et al., 1992).

We have also measured the catalytic activity of NF1-333 with increasing concentrations of p21-GTP (Fig. 3). On the assumption that p21-GTP is the substrate and NF1-333 is the enzyme we obtain values for $k_{\text{cat}}$ and $K_M$, which are different from the values obtained for p120-GAP (Table I). The maximal rate of GTP hydrolysis in the presence of NF1-333 is $1.4 \text{s}^{-1}$. Since the uncatalyzed GTP hydrolysis reaction of p21 is $1.2 \times 10^{\text{-}4} \text{s}^{-1}$ under the same conditions, it means that the reaction is stimulated $1.1 \times 10^{\text{5}}$-fold. The stimulation is thus about 10-fold lower as compared with full-length GAP, and the $K_M$ is also much lower. The higher affinity of p21 for NF1-GRD and the lower catalytic potential have been noted before with recombinant protein obtained from baculovirus (Martin et al., 1990). The $K_M$ value reported here agrees very well with the $K_D$ value measured by inhibition studies (Martin et al., 1990). It should also be mentioned that the $k_{\text{cat}}/K_M$ value, the specificity constant for the enzymatic reaction, is $4.7 \times 10^{\text{5}} \text{M}^{-1} \text{s}^{-1}$ for NF1-333 and thus very similar to that for p120-GAP, but much higher than for GAP-334. Contrary to what is found for p120-GAP it appears that in NF1-GAP the catalytic domain is sufficient for full activity, unless it turns out that full-length NF1-GAP has an even higher activity than full-length p120-GAP.

Fig. 1 shows the totally conserved amino acids from a region of the catalytic domain that is the most highly conserved. It has been speculated that the GTPase reaction in the presence of GAP might have a mechanism different from that which has been proposed for the uncatalyzed GTPase reaction (Wittinghofer and Pai, 1991; Pai et al., 1990). It could be envisioned that GAP supplies additional catalytic residues for improved GTP hydrolysis. Potential candidates for such catalytic residues could be the totally conserved arginine and lysine residues shown in Fig. 1. Arginine in p120-GAP has already been shown to be involved in catalysis (Skinner et al., 1991). Here we mutated Lys-1422 to Met (for nomenclature see Gutmann et al., 1991) and investigated the properties of the mutant protein NF1-333(K1422M). The protein was purified by the same procedure as wild-type protein and was found to elute under the same conditions from chromatographic columns. The results of activity measurements were found to be irreproducible. The specific activity varied between 26 and 1443 units/mg. The reason for this can be seen in Fig. 4, which shows the inactivation with temperature of wild-type and mutant protein. Although an exact melting temperature has not been determined it is clear that the thermal stability of NF1-333(K1422M) is greatly reduced, the difference in the...
inactivation temperature of the catalytic activity being 40 °C. Thus it is not surprising that during different preparations of the protein different amounts of protein are inactivated.

Another indication of the stability of the protein comes from protease digestion experiments shown in Fig. 5. It shows that both proteins are readily digested by trypsin under native conditions to a polypeptide of approximately 31 kDa. However, the Lys-1422 mutant is digested much faster to a smaller fragment with 29 kDa with a half-life of less than 30 min, whereas the half-life for the wild-type protein is around 240 min.

It has been noted before that the GTPase activating activity of GAP is very sensitive to salts (McCormick et al., 1988; Gibbs et al., 1988). A remarkable difference between wild-type and mutant NF1-333 is the much higher sensitivity of the catalytic activity of the mutant protein toward sodium chloride (Table I). With 50 mM NaCl 50% of the activity is lost, whereas wild-type protein has an IC50 value of 200 mM. The catalytic activity of NF1-333 is thus less sensitive to salts than that of p120-GAP (McCormick et al., 1988; Gibbs et al., 1988).

From these experiments it appears that the totally conserved lysine residue is important for the thermal stability of the GAP proteins. It seems likely that the side-chain amino group is not free on the surface and available for interaction with p21-GTP, but rather stabilizes the protein via an internal hydrogen bond or an ionic interaction.

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REFERENCES