Expression of a lipocalin in *Pichia pastoris*: secretion, purification and binding activity of a recombinant mouse major urinary protein

E. Ferrari[a], T. Lodi[b], R.T. Sorbi[c], R. Tirindelli[c], A. Cavaggioni[d], A. Spisni[a,*]

[a]*Istituto di Chimica Biologica, Facoltà di Medicina e Chirurgia, Università degli Studi di Parma, via Gramsci 14, I-43100 Parma, Italy*
[b]*Istituto di Genetica, Università di Parma, viale delle Scienze, Parma, Italy*
[c]*Istituto di Fisiologia Umana, Università di Parma, via Gramsci 14, Parma, Italy*
[d]*Istituto di Fisiologia Umana, Università di Padova, via Marzolo 8, Padua, Italy*

Received 4 November 1996; revised version received 25 November 1996

Abstract The proteins of the mouse major urinary protein complex (MUP), members of the lipocalin family, bind volatile pheromones and interact with the vomeronasal neuroepithelium of the olfactory system. We report the expression of a MUP protein using its native signal sequence for secretion in the methylotrophic yeast, *Pichia pastoris*. Mature recombinant MUP (rMUP) is secreted at a concentration of 270 mg/l in minimal medium and it is isolated from the culture supernatant by one step ion-exchange chromatography in a nearly pure form. Binding activity, tested with an odorant molecule which displays high affinity for native MUP, indicates that rMUP has a behavior similar to the native one. This finding suggests that the protein, and in particular its hydrophobic binding pocket, is properly folded.

Key words: Major urinary protein; *Pichia pastoris*; Heterologous expression

1. Introduction

An important component of adult male mouse urine is the major urinary protein complex (MUP), whose biological function relates to olfaction and sexual communication. MUP is the expression product of a large gene family located on chromosome 4 [1]. Transcription of this gene family takes place in liver cells under a complex endocrine control, dependent on androgens, so that only sexually mature male mice excrete MUP in amounts in the order of 1–5 mg/ml [2]. MUP belongs to the lipocalin superfamily [3,4], of which many members bind hydrophobic ligands with high affinity and selectivity forming long-lasting complexes [5]; the family is typified by the retinol binding protein of serum [6–9]. The MUP binding process takes place in the blood plasma; subsequently, MUP is concentrated in the urine and, once excreted, it slowly releases into the air volatile molecules, most of which have been isolated, identified [10,11] and characterized by their hormonal and behavioral activities. In addition, in vivo experiments point to a pheromonal role of the protein free of ligands [12]. It consists in the ability to stimulate the vomeronasal section of the female mouse olfactory system [13,14], thus priming hormonal responses.

*Corresponding author. Fax: (39) (521) 988952.
E-mail: aspin@ipruniv.cce.unipr.it

Abbreviations: rMUP, recombinant major urinary protein; *AOX1*, alcohol oxidase 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; [^{3}H]IBMP, [^{3}H]2-isobutyl-3-methoxypyrazine

Here we report the use of the methylotrophic yeast *Pichia pastoris* as host for the expression of homogeneous and functional recombinant MUP (rMUP). A unique feature of this expression system is the promoter employed to drive heterologous gene expression, which is derived from the methanol-regulated alcohol oxidase 1 gene (*AOX1*) of *P. pastoris* [15–17]. *AOX1* regulation is achieved at the transcriptional level: the gene is efficiently transcribed in cells exposed to methanol as sole carbon source and repressed in alternative carbon sources such as glucose and glycerol. Therefore, under the control of the *AOX1* promoter, the MUP coding gene was maintained in an 'expression-off' mode on glycerol (cell growth phase) and switched on by shifting to methanol (expression phase). To maximize the stability of the production strains, an expression cassette, assembled in pHIL-D2 vector and containing the MUP cDNA under the promoter, was integrated into the host genome. To this purpose, the cassette was opportunely flanked by *AOX1* terminal sequences that are known to favor gene replacement at the *AOX1* locus. Following transformation, strains slowly metabolizing methanol turned out to be the ones deleted at *AOX1*, relying only on the expression of a second but transcriptionally weak alcohol oxidase gene, *AOX2*. This phenotype, 'methanol utilization slow' (Mut^s), was successfully used to characterize, among all the transformants, the clones harboring a functional expression cassette at the *AOX1* locus. All the selected Mut^s clones were shown to secrete rMUP in minimal salt medium.

2. Materials and methods

2.1. Strains and transformations

E. coli strain XL-1 Blue (Stratagene) was used for transformation and propagation of the recombinant plasmids. *P. pastoris* strain GS115 (*his4*) was used in the expression study. Yeast transformation was performed according to the electroporation method described in the Manual Version 3.0 of the Pichia Expression Kit (Invitrogen, San Diego, CA).

2.2. cDNA clone isolation and construction of the expression plasmid

MUP cDNA clones were isolated from total mRNA obtained from adult male mouse liver by RT-PCR, using the following primers: oligo1: 5′-CCTCCATGGATGAAGATGCTGCTGCTG-3′, and oligo2: 5′-TCAAACACACTGCAGGCTCAGGCC-3′. The selected MUP cDNA was ligated into the pHIL-D2 vector (Invitrogen) at the *Eco*RI site to yield the vector pHIL-D2MUP.

Plasmid construction was performed using established techniques [18] and cloning steps were ensured by both strand DNA sequencing (Sequenase version 2.0, USB).

MUP cDNA integration in *P. pastoris* recombinant genomes was confirmed by genomic PCR carried out with the two primers: 5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCTGACATCC-3′, referred to as 5′ *AOX1* and 3′ *AOX1* primers,

PII S0014-5793(96)01436-6

respectively. Total DNA isolation from *P. pastoris* was performed as described by Ausubel [19].

2.3. Yeast cultures

Cultures were grown in an inorganic salt-based medium (mineral minimal medium) consisting of the following components: 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 3.5 g/l $(NH_4)_2SO_4$, 0.1 g/l NaCl, 0.1 g/l $CaCl_2$, 0.5 mg/l H_3BO_3, 0.1 mg/l KI, 0.2 mg/l $FeCl_3$, 0.4 mg/l $MnSO_4$, 0.4 mg/l $FeSO_4$, 0.2 mg/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 10 mg/l biotin and either 1% glycerol or 0.5% methanol. Potassium phosphate buffer pH 7.2 was added at a final concentration of 100 mM to maintain the neutral pH during the whole expression time. Transformants were scored for their methanol utilization phenotype: Mut^s strains were distinguished from the other ones by patching on minimal dextrose versus minimal methanol (without dextrose) plates.

2.4. Screening for rMUP expression

His^+ Mut^s transformants were inoculated in 25 ml of buffered mineral minimal medium with glycerol and grown in a flask agitator at 28°C for about 24 h. At a density of about 2.5×10^8 cells/ml (5 OD 600 Unit) cells were collected and resuspended at a density of about 7.5×10^8 cells/ml in the same medium, this time containing methanol as the sole carbon source. The induction period was 6 days, with regular pulses of methanol to keep the inducer concentration at 0.5%. The supernatants were collected by centrifugation ($4000\times g$, 10 min) and the amounts of secreted protein were quantitated by the Bio-Rad protein assay (Bio-Rad) with natural MUP as a standard.

2.5. Purification and characterization of rMUP

The culture supernatant was chilled and clarified by centrifugation ($12\,000\times g$, 15 min). The solution was equilibrated with 10 mM Tris-HCl buffer at pH 7.2, by dialysis at 4°C, using a dialysis tube with 15 000 Da cut off (Servapor). Purification was achieved using FPLC with a SourceQ column (Pharmacia) and an NaCl gradient in 10 mM Tris-HCl buffer at pH 7.2. Fractions with rMUP were identified spectrophotometrically ($\varepsilon_{1\%}^{276nm}=6.3$, as reported by Cavaggioni [20]) and pooled. SDS-PAGE electrophoresis was carried out on 15% acrylamide gel and Coomassie blue or silver staining was used. N-terminal sequencing was performed on an automated protein sequencer (Applied Biolab) with a standard protocol of Edman degradation. Molecular mass was determined by time of flight mass spectrometry in a MALDI equipment, using sinapinic acid as the light absorbing matrix.

Immunological competence was tested by dot blot on nitrocellulose filters using the Vectastain ABC Kit (Vector Labs, Burlingame, CA) and polyclonal antibodies raised in rabbit against natural MUP purified from male mouse urine.

2.6. Ligand binding test

This test takes advantage of the volatility of the ligand [^{3}H]2-isobutyl-3-methoxypyrazine ([^{3}H]IBMP) allowing it to diffuse from a solution placed at the bottom of a Petri dish into drops of buffer solution, with and without rMUP, hanging down from the cover. The binding reaction P+L↔B, where P = rMUP, L = [^{3}H]IBMP and B is the ligand (L) bound to the protein, was studied at equilibrium. The tritiated radioligand [^{3}H]IBMP with a specific activity of 40 Ci/mmol was obtained by tritiating the dehydroisobutyl precursor (courtesy of Dr. P. Pelosi, Pisa University). Nine Petri dishes (9 cm in diameter) were half filled with 20 ml of a 10 mM phosphate buffer, 10 mM NaCl, pH 7.2, an increasing concentration of cold IBMP and a fixed concentration of [^{3}H]IBMP (10^4 cpm/ml). Five 50 μl drops of 26 μM rMUP solution in 10 mM phosphate buffer pH 7.2, 10 mM NaCl and three control drops without the protein were placed near the center of each cover. To avoid spreading of the hanging drops the glass of the cover was made hydrophobic by silanization. The covers were then placed on top of the dishes to complete the diffusion chamber (Fig. 1) and equilibration of [^{3}H]IBMP was obtained in 40 h. Radioactivity of each drop was counted individually with 3 ml of liquid scintillator. The total concentrations of the ligand [B+L] in the drops containing rMUP and of the free ligand [L] in the drops without rMUP were calculated from their mean radioactivity on the basis of [^{3}H]IBMP specific activity. Assuming that, at equilibrium, the free ligand concentration [L] is identical in the drops with and without rMUP, the concentration of the bound ligand, [B], was then determined as:

$$[B] = [B + L] - [L]$$

From the Scatchard equation:

$$B/L = -1/K_m(B - B_{max})$$

the dissociation constant K_m and the maximum concentration of bound ligand B_{max} were obtained with a S.D./mean = 0.2.

3. Results and discussion

3.1. Isolation of MUP cDNA and pHIL-D2MUP vector assembling

Total mRNA was obtained from the liver of an adult male mouse where MUP mRNAs are known to account for 5–10% of total mRNA [21,22]. Various MUP cDNA clones were amplified in agreement with the notion that several MUP genes are transcribed at the same time [23]. The cDNA selected for expression reported in Fig. 2A corresponds to MUP type IIa cDNA [21,24] and MUP BL1 gene [25].

rMUP secretion from *P. pastoris* (GS115 strain) was performed using pHIL-D2 vector specifically designed for intracellular heterologous expression, as it does not contain a yeast secretion signal. The cDNA sequence, including the native signal peptide and a Kozak sequence, introduced by PCR, was ligated into pHIL-D2 vector giving rise to pHIL-D2MUP. The AAG consensus sequence for proper translation initiation [26] was based on the trinucleotide biases observed in yeast at the −3, −2 and −1 positions upstream of the start codon. pHIL-D2MUP plasmid was then linearized with *Not*I in order to give an integrative fragment containing the MUP cDNA and the *HIS4* selectable marker. The plasmid was then used to transform *P. pastoris* by electroporation. This segment, opportunely flanked by sequences originating from regions 5′ and 3′ of the *AOX1* gene, directed the MUP cDNA to *P. pastoris* genome by means of a recombination event at the *AOX1* locus. When this integration replaced the *AOX1* gene, transformant strains metabolizing methanol at a reduced rate (Mut^s phenotype) were generated (Fig. 2B). His^+ transformants were selected on mineral minimal medium and tested for methanol utilization. Nine Mut^s clones were analyzed by PCR amplification of the genomic region encompassed by 5′ and 3′ TT (transcription termination) *AOX1* sequences, with primers annealing to 5′ and 3′ TT *AOX1* regions; the reaction confirmed that the MUP expression cassette substituted the wild-type *AOX1* gene (Fig. 3A).

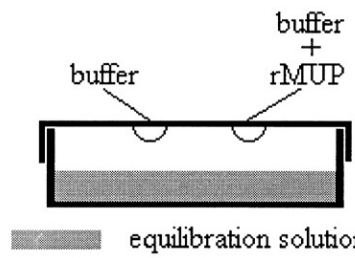

Fig. 1. Diffusion chamber used for rMUP binding studies. The equilibration solution, placed at the bottom of a Petri dish, is the reservoir of the ligand [^{3}H]IBMP; several drops of buffer solution with and without rMUP are hanging down from the cover. The volatile ligand diffuses in the space above the solution until equilibrium is reached, i.e. the free ligand dissolves in all drops to the same concentration as the reservoir.

3.2. Methanol-regulated expression and secretion of rMUP

The selected Mut^s recombinants were screened for expression levels by determining the amount of rMUP secreted in the extracellular medium, as described in Section 2. Samples of the expression medium supernatants, taken at various time intervals, were analyzed by SDS-PAGE to determine the optimal induction time. The recombinant protein only, migrating with native MUP at about 20 kDa, was detectable by Coomassie blue staining. The electrophoretic profile (Fig. 3B) reveals the protein regularly accumulating up to about 0.2 mg/ml over an expression period of 6 days. Clone MN6 secreted rMUP in the largest amount, reaching the concentration 0.27 mg/ml at the end of the induction period. Cell concentration in the expression phase was stable at 7.5×10^8 cells/ml (14 g dry cells/l). The expression volume has been scaled up to 100 ml per flask revealing a comparable accumulation of rMUP. There was no proteolysis of rMUP, hence it was not necessary to supplement the medium with casamino acid or peptone as excess substrate for proteases.

3.3. Characterization of rMUP and ligand binding test

FPLC of the expression supernatants showed the protein to be eluted as a single peak at relatively high ionic strength (Fig. 4A), in agreement with its acidic nature (pI = 4.55).

N-terminal sequence analysis of the first 14 residues gave the correct sequence H_2N-EEASSTGRNPNVEK-, indicating that the pre-protein was properly recognized and processed by *P. pastoris* secretion machinery.

rMUP reacted with polyclonal antibodies raised in rabbit against purified natural MUP; controls without primary antibody and with preimmune serum were negative.

The molecular weight of rMUP was defined by mass spectrometry and was 18749 instead of 18710 as expected [27]. Such a mass difference may be due to a covalent modification of some residues, e.g. methylation or oxidation.

A functional characterization of rMUP was carried out by testing its ability to bind the ligand [^{3}H]IBMP, which is known to interact with the native MUP [10,20]. The binding isotherm (18°C) of rMUP is reported in Fig. 4B. The results indicate that the total ligand concentration in the equilibration solution did not limit the protein binding. rMUP was found to bind [^{3}H]IBMP with a dissociation constant (K_m) of 0.94 μM, in agreement with the value reported by Bacchini et al. [10], and a $B_{max} = 0.88$. The latter binding parameter

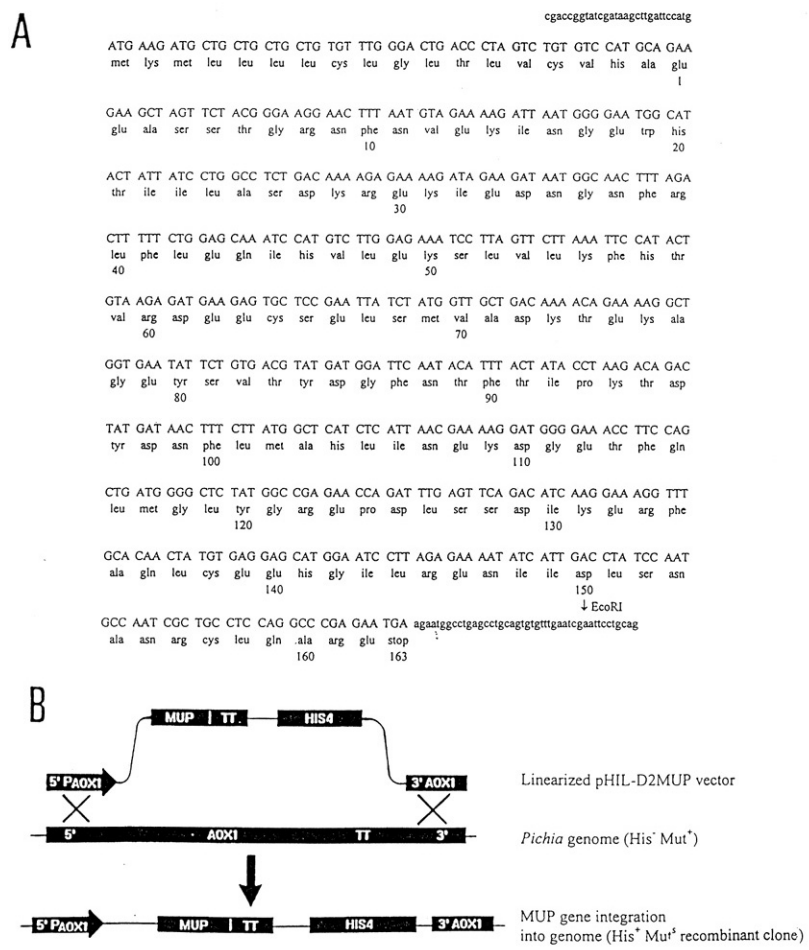

Fig. 2. A: Nucleotide sequence of MUP cDNA together with the deduced amino acid sequence. B: Gene replacement at *AOX1*. The linearized pHIL-D2MUP vector is composed of a plasmid-borne version of the *AOX1* promoter (5′ *AOX1*), the MUP cDNA, the transcription termination fragment (TT), the *HIS4* gene for selection of transformants and the 3′ *AOX1* sequence from *AOX1* gene. Cleavage of the vector within 5′ *AOX1* and 3′ *AOX1* sequences, shared by the host genome, stimulated homologous recombination events which efficiently integrated the MUP expression cassette into the *P. pastoris* host genome, removing the *AOX1* coding region. The resulting His$^+$ integrants metabolized methanol at a reduced rate (phenotype Mut^s).

differs from the one of the native MUP, $B_{max} = 0.3$ [20]. This discrepancy could be due to the presence of isoforms in the native MUP [28] that might be inactive or preferentially interacting with others ligands [11] thus reducing the binding capacity of the native MUP complex toward [^{3}H]IBMP. Studies are under way to verify these last two points.

Finally, preliminary data from the deconvolution of CD spectra (in preparation) show that the structural elements of rMUP and of native MUP are quite similar.

4. Conclusion

The major urinary protein complex of mice is involved in pheromonal communication among conspecifics and the understanding of its structure-function relationship can be quite relevant to describe the complex physiological processes associated with its activity.

Unfortunately, though it is present in large amounts in the urine of mature mice, it is expressed in several isoforms difficult to separate [28].

The main result of this work is that a protein of the male mouse MUP complex has been expressed in the *P. pastoris* yeast host system with proper folding and with binding properties comparable to the native one. To our knowledge, this is the first report of a protein of the lipocalin family being expressed in quantitative yields, with proper fold and activity.

As a consequence of the low levels of secretion of *P. pastoris* endogenous proteins, rMUP appeared to be the only protein in the culture broth, an advantage in processing and purification. Simple growth medium and small-scale cultures were the only requirements to obtain functional rMUP.

Protein secretion was achieved using the native signal peptide, confirming that the yeast secretion machinery is competent to process mammalian secretory proteins [16]. This result makes it conceivable to express, with a similar strategy, other members of the lipocalin superfamily to the advantage of physiological and structural investigations.

Finally, as the expression in minimal medium is suited to enrich protein in ^{13}C as well as in ^{15}N with [^{13}C]methanol and

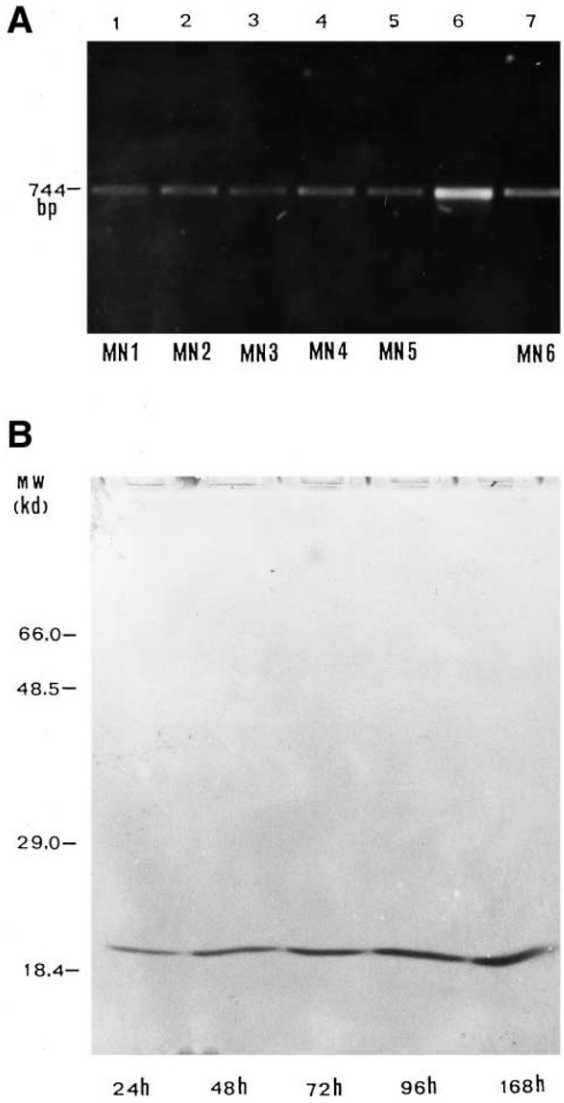

Fig. 3. A: PCR amplifications of the genomic region between 5′ and 3′ TT *AOX1* sequences in six representative transformants, MN1, MN2, MN3, MN4, MN5, MN6. Lane 6 shows the amplification positive control, i.e. the 744 bp PCR product (including the MUP coding segment) of the region limited by the same *AOX1* sequences in the pHIL-D2MUP vector. The MUP cDNA is integrated in all tested Mut^s recombinants. The wild-type *AOX1* gene (2.2 kb) is absent because replaced by the MUP expression cassette. B: SDS-PAGE analysis indicating that rMUP is accumulated in the expression culture of clone MN2 during an induction cycle: 15 μl of medium, collected at the indicated induction times, was loaded on the gel. Staining with Coomassie blue detected rMUP only.

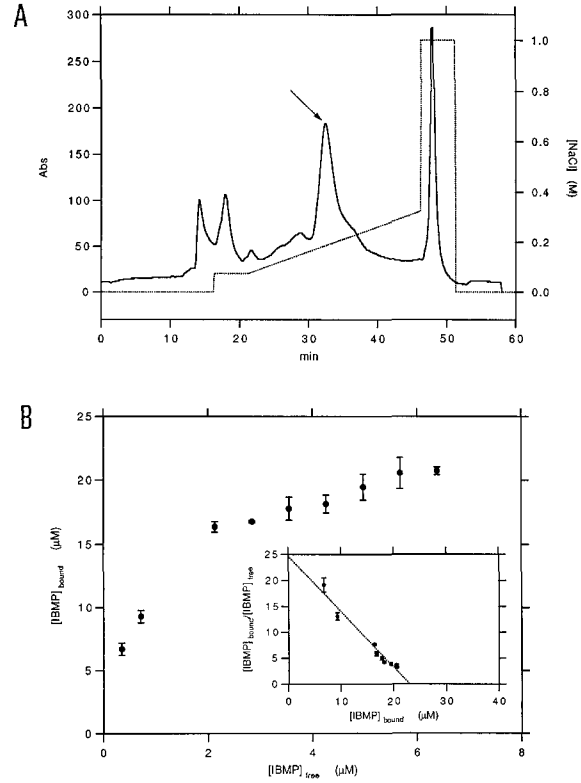

Fig. 4. A: Chromatogram of rMUP separation from the supernatant by ion exhange chromatography. The expression supernatant was chromatographed on a Source Q column and rMUP was eluted with a NaCl gradient (dashed line) in Tris-HCl 10 mM pH 7.2. The arrow indicates the rMUP elution peak. B: Binding isotherm (18°C) of the odorant IBMP for rMUP. X-axis: free IBMP concentration. Y-axis: bound IBMP concentration. Scatchard analysis (inset) indicates a single set of binding sites of $K_m = 0.94$ μM and a $B_{max} = 0.88$.

[^{15}N]ammonium sulfate, respectively, we are now in the process of obtaining a uniformly labelled protein for NMR structure determination studies. Structural studies of the protein in solution by means of multinuclear NMR spectroscopy, combined with site-directed mutagenesis techniques, will help in interpreting the relationship between structure and biological function of this protein.

Acknowledgements: The work was partly supported by MURST (40% and 60%) and by CNR Grant No. 95.02939.14/115.19363. The authors are indebted to Dr. Laura Perantoni for valuable help in carrying out the binding experiments. The Interfaculty Center of Measurements (CIM) of the University of Parma is acknowledged for the use of the PCR, CD and computing facility.

References

[1] Clark, A.J., Ghazal, P., Bingham, R.W., Barrett, D. and Bishop, J.O. (1985) EMBO J. 12, 3159–3165.
[2] Clissold, P.M., Hainey, S. and Bishop, J.O. (1984) Biochem. Genet. 22, 379–387.
[3] Flower, D.R., North, A.C.T. and Attwood, T.K. (1993) Protein Sci. 2, 753–761.
[4] Flower, D.R. (1995) J. Mol. Recogn. 8, 185–199.
[5] Pelosi, P. (1995) Crit. Rev. Biochem. Mol. Biol. 29, 199–228.
[6] Cowan, S.W., Newcomer, M.E. and Jones, T.A. (1990) Proteins Struct. Funct. Genet. 8, 44–61.
[7] Branden, C. and Tooze, J. (1991) Antiparallel β-structures. In: Introduction to Protein Structure. Garland, New York.
[8] Sivaprasadarao, A., Boudjelal, M. and Findlay, J.B.C. (1993) Biochem. Soc. Trans. 21, 619–622.
[9] Zanotti, G., Marcello, M., Malpeli, G., Folli, C., Sartori, G. and Berni, R. (1994) J. Biol. Chem. 26, 29613–29620.
[10] Bacchini, A., Gaetani, E. and Cavaggioni, A. (1992) Experientia 48, 419–421.
[11] Robertson, D.H.L., Beynon, R.J. and Evershed, R.P. (1993) J. Chem. Ecol. 19, 1405–1416.
[12] Mucignat-Caretta, C., Caretta, A. and Cavaggioni, A. (1995) J. Physiol. 486, 517–522.
[13] Kaneko, N., Debski, E.A., Wilson, M.C. and Whitten, W.K. (1980) Biol. Reprod. 22, 873–878.
[14] Keverne, E.B. (1983) Trends Neurosci. 6, 381–384.
[15] Cregg, J.M., Tschopp, J.F., Stillman, C., Siegel, R., Akong, M., Craig, W.S. et al. (1987) Bio/Technology 5, 479–485.
[16] Digan, M.E., Lair, S.V., Brierley, R.A., Siegel, R.S., Williams, M.E., Ellis, S.B. et al. (1989) Bio/Technology 7, 160–164.
[17] Cregg, J.M., Vedvick, T.S. and Raschke, W.C. (1993) Bio/Technology 11, 905–910.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[19] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1990) Current Protocols in Molecular Biology, Greene/Wiley-Interscience, New York.
[20] Cavaggioni, A., Findlay, J.B.C. and Tirindelli, R. (1990) Comp. Biochem. Physiol. 96B, 513–520.
[21] Shahan, K., Gilmartin, M. and Derman, E. (1987) Mol. Cell. Biol. 7, 1938–1946.
[22] McIntosh, I. and Bishop, J.O. (1989) Mol. Cell. Biol. 9, 2202–2207.
[23] Clissold, P.M. and Bishop, J.O. (1982) Gene 18, 211–220.
[24] Johnson, D., Al-Shawi, R. and Bishop, J.O. (1995) J. Mol. Endocrinol. 14, 21–34.
[25] Clark, A.J., Chave-Cox, A., Ma, X. and Bishop, J.O. (1985) EMBO J. 4, 3167–3171.
[26] Cavener, D.R. and Stuart, C.R. (1991) Nucleic Acids Res. 19, 3185–3192.
[27] Evershed, R.P., Robertson, D.H.L., Beynon, R.J. and Green, B. (1993) Rapid Commun. Mass Spectrom. 7, 882–886.
[28] Robertson, D.H.L., Cox, K.A., Gaskell, S.J., Evershed, R.P. and Beynon, R.J. (1996) Biochem. J. 316, 265–272.