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Biochemistry

Arginine in the leader peptide is required for both import and proteolytic cleavage of a mitochondrial precursor

(mitochondrial protein targeting/leader peptide/oligonucleotide-directed mutagenesis/arginine residues/ornithine transcarbamoylase)

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ABSTRACT **Most mitochondrial proteins are encoded in the nucleus and translated in the cytoplasm as larger precursors containing NH_2-terminal "leader" peptides, which are strikingly basic in overall amino acid composition. Recent experiments indicate that these leader peptides are both necessary and sufficient to direct post-translational recognition and import of precursors by mitochondria. In this report, we demonstrate a critical role for one or more of the basic arginine residues in the leader peptide of the subunit precursor for the human mitochondrial matrix enzyme, ornithine transcarbamoylase (ornithine carbamoyltransferase, carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3). The distal three of four basic residues, all arginines, in the leader peptide of ornithine transcarbamoylase were replaced at once with charge-neutral glycine residues. The altered ornithine transcarbamoylase precursor failed to be taken up by intact mitochondria *in vitro*. Moreover, it also failed to be proteolytically cleaved upon incubation with a mitochondrial matrix fraction containing the Zn^{2+}-dependent protease, which normally cleaves the leader peptide.**

Many cellular proteins reside in compartments remote from their cytoplasmic site of synthesis. Recent studies have focused on the mechanisms by which newly synthesized proteins reach these compartments. A number of studies have identified specific amino acid sequences within the newly synthesized proteins that are responsible for targeting the proteins to their particular destinations (1–3). In the case of proteins destined for mitochondria or their plant cell analogue, chloroplasts, the primary translation products generally are precursors containing NH_2-terminal leader peptides. These leaders range in size from 20 to 60 amino acid residues and are cleaved post-translationally after entry into the organelle (3, 4). Recently, three different leader peptides—one each from pea (5), yeast (6), and man (7)—have been shown in gene fusion experiments to contain sufficient information to direct import by mitochondria or chloroplasts of chimeric proteins composed of a leader peptide of a mitochondrial or chloroplast precursor joined with a polypeptide chain that normally localizes to the cytosol.

How do leader peptides carry out their targeting function? To approach this question, we and others have compared the available sequences in an effort to identify elements that are shared and potentially functional. No extensive homology among these sequences has been found. Importantly, however, all of the dozen leader peptides analyzed to date are essentially devoid of acidic residues and contain an average to above-average content of basic residues (3, 8). This strikingly basic overall composition predicts a net positive charge for the leader domain, which is likely to contribute to its function.

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In a previous study, we analyzed the leader peptide of the subunit precursor of the human mitochondrial matrix enzyme ornithine transcarbamoylase (OTCase; ornithine carbamoyltransferase, carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) (8). The OTCase leader peptide contains 32 residues and resembles in composition the other leader peptides analyzed to date (it lacks acidic residues and contains 4 arginine residues; see Fig. 1*A*). Recently, we assessed the role of arginine residues in import of the OTCase precursor. Cultured cells expressing a transfected and amplified OTCase cDNA were incubated in medium in which arginine was replaced with its charge-neutral analogue, canavanine (9). The canavanine-substituted OTCase precursor failed to be converted to the mature-sized subunit, either because it could not be imported by mitochondria or because it was resistant to cleavage by the ubiquitous Zn^{2+}-dependent mitochondrial matrix protease. We could not distinguish between these two effects in the intact cell system, nor could we exclude the possibility that the observed effect resulted from substitution of one or more of the 12 arginine residues in the mature portion of the OTCase precursor rather than from an effect on the leader. Therefore, to directly address the role of arginine in the OTCase leader peptide, we have programmed expression of an altered OTCase precursor in which the distal 3 arginine residues within the leader peptide have been replaced at the same time with glycine residues. We report here that the altered precursor fails both to be imported and to be cleaved proteolytically.

MATERIALS AND METHODS

Material. Restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs. RNasin was obtained from Promega Biotech (Madison, WI), and SP6 RNA polymerase was from New England Nuclear. [^{35}S]-Methionine was obtained from Amersham.

Synthetic Oligonucleotides. DNA fragments were synthesized on a solid substrate using an Applied Biosystems 380A synthesizer, and they were purified in acrylamide/urea gels after deblocking.

Plasmids. Plasmid pSPOTC2 contains the entire human OTCase coding sequence and portions of the untranslated regions, inserted into the *Pst* I polylinker site in plasmid pSP64. The plasmid was derived by excision of an *Eco*RV/*Sma* I fragment from pSPOTC1 (9), removing a *Bam*HI site from the polylinker and leaving a single *Bam*HI site in the OTCase leader coding sequence.

***In Vitro* Transcription.** Plasmid DNA containing an SP6 promoter sequence joined with a cloned coding sequence was prepared for transcription by restriction cleavage at a unique *Sac* I site downstream from the coding sequence. One micro-

Abbreviation: OTCase, ornithine transcarbamoylase.

gram of linearized plasmid was then used in a transcription reaction performed according to Krieg and Melton (10).

***In Vitro* Translation and Incubations with Isolated Mitochondria or a Mitochondrial Matrix Fraction.** Cell-free protein synthesis was carried out in a nuclease-treated rabbit reticulocyte lysate system as described (11). The ethanol-precipitated products of an *in vitro* transcription reaction were resuspended in 30 μl of water; 3 μl was added to 20 μl of reticulocyte lysate containing 30 μCi of [^{35}S]methionine (1 Ci = 37 GBq), and the mixture was incubated for 1 hr at 30°C, following which 9 μg of cycloheximide was added. For direct analysis, 2 μl of the translation products were immunoprecipitated using anti-rat OTCase antiserum. For mitochondrial incubation, 10 μl of the translation products were combined with 1 μl of a suspension of freshly isolated rat liver mitochondria (40 mg/ml) and incubated at 27°C for 1 hr. The mitochondrial mixture was then either separated into supernatant and mitochondrial fractions by centrifugation at 10,000 × *g* for 6 min, or first digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at a trypsin/total protein ratio of 1:250 for 10 min at 27°C and then separated. A mitochondrial matrix fraction was prepared by the method previously described (12). Ten microliters of the translation products was mixed with 2 μl of the matrix fraction (total protein, 8 mg/ml) and with 1 μl of 1.3 mM $ZnCl_2$ and incubated at 27°C for 1 hr. Products of the mitochondrial and matrix incubations were immunoprecipitated with anti-rat OTCase antiserum by the method previously described (13).

RESULTS

Construction of a Plasmid Encoding an Altered OTCase Precursor. To produce an altered OTCase precursor, a cloned cDNA sequence was constructed to program its expression. As a first step, a set of oligonucleotides was synthesized that encoded the portion of the precursor to be altered. As shown in Fig. 1*A*, we synthesized a group of five oligonucleotides that could be annealed to form an 89-base-pair *Bam*HI/*Pvu* II fragment. The fragment encodes residues 8–36 of the OTCase precursor, comprising residues 8–32 of the leader, followed by the first four residues of the mature OTCase subunit. The sequence is identical to that of the wild-type OTCase precursor at all positions except the three designated with boxes in Fig. 1*A*, where three arginine codons present in the wild-type sequence have been changed to glycine codons in the mutant segment. Thus, while the wild-type OTCase leader peptide contains arginine at positions 6, 15, 23, and 26, the altered OTCase precursor is predicted to contain arginine only at residue 6. The mixture of synthetic oligonucleotides was annealed, incubated with T4 DNA ligase, and then joined with the remainder of the wild-type OTCase coding sequence and the *Salmonella* phage promoter, SP6, to form the plasmid pSPmLOTC, as illustrated in Fig. 1*B*. The region in pSPmLOTC coding for the leader peptide was subjected to DNA sequence analysis; the predicted sequence was confirmed throughout.

Synthesis of Altered Precursor and Incubation with Isolated Mitochondria. Previous studies have shown that when a rabbit reticulocyte lysate-programmed translation mixture containing radiolabeled mitochondrial precursors is incubated with intact rat liver mitochondria, the precursors are taken up and proteolytically cleaved to their mature forms (3, 11). Both the altered and wild-type OTCase precursors were studied by using this system. Using SP6 polymerase, RNA encoding the respective precursors was transcribed *in vitro* from plasmid pSPmLOTC and from a homologous plasmid containing the wild-type sequence (pSPOTC2). The transcription products were then incubated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Portions of the translation products were directly immunoprecipitated with anti-OTCase antiserum. As shown in Fig. 2, major precipitable products were detected corresponding to altered (lane 6) and wild-type (lane 1) precursors; these migrated identically. The remaining portions of the translation reactions were incubated with isolated rat liver mitochondria for 1 hr at 27°C, and the mixtures were then separated by centrifugation into supernatant and mitochondrial pellet fractions. The fractions were solubilized and immunoprecipitated. As shown in Fig. 2 (lanes 7 and 8), virtually all of the altered precursor remained in the supernatant (lane 7); only a small amount sedimented with the mitochondria (lane 8). No mature-sized species was seen. The latter finding could be the result either of failure of the precursor to be imported by the mitochondria, or of an inability of the altered protein, following proper import, to be cleaved by the matrix protease. Failure of import is strongly suggested by two observations. First, only a small fraction of the radiolabeled precursor was found with the mitochondrial pellet (lane 8). In contrast, when the wild-type precursor was incubated with mitochondria (lanes 2 and 3), a substantial fraction of the radiolabeled precursor was converted to the mature form and sedimented with the mitochondrial pellet (lane 3).

Second, none of the altered precursor associated with the mitochondria was resistant to treatment with trypsin. In these experiments, the mitochondrial import mixture was treated with a low concentration of trypsin prior to separation into supernatant and pellet fractions. Such trypsin treatment resulted in complete proteolytic degradation of the altered precursor in both the supernatant (lane 9) and mitochondrial pellet (lane 10) fractions. In the wild-type analysis, the mature OTCase subunit was protected from the action of exogenous trypsin (lane 5), consistent with its intramitochondrial localization. Wild-type precursor in the supernatant (lane 4) or pellet (lane 5) was completely degraded by such treatment, however, implying that the OTCase precursor associated with mitochondria was on the outside of the organelle.

Incubation of Altered Precursor with Mitochondrial Matrix Fraction Containing Leader Protease. Finally, we incubated the *in vitro*-synthesized precursors with a mitochondrial matrix fraction containing the Zn^{2+}-dependent enzyme that normally cleaves the leader peptide (12, 14). As shown in Fig. 3 (lane 2), when the wild-type precursor was incubated with the matrix fraction, cleavage to the mature form was readily detected. When the altered precursor was incubated under identical conditions, no proteolytic cleavage was detected (lane 4). Varying neither the concentration of mitochondrial protein nor the concentration of Zn^{2+} resulted in observable cleavage of the altered precursor. Therefore, we conclude that the altered leader peptide fails to be recognized and/or acted upon by the protease.

DISCUSSION

Effects of Substitution of Glycine for Arginine in the OTCase Leader Peptide. The simultaneous substitution of glycine for arginine at three positions in the leader peptide of the OTCase precursor results in apparent failure of the altered precursor to be imported by mitochondria and in failure of the leader peptide to be cleaved by the matrix protease. We asked whether these two effects might be related. On the one hand, failure of entry of the mutant precursor, as indicated by its sensitivity to exogenous trypsin, would result in a lack of cleavage, because the precursor would be unable to reach the matrix compartment where the protease resides. On the other hand, failure of cleavage of a properly imported precursor could conceivably produce the appearance of failure of import if the altered precursor was not retained within mitochondria. This latter possibility has been addressed by Zwizinski and Neupert (15), who demonstrated accumulation

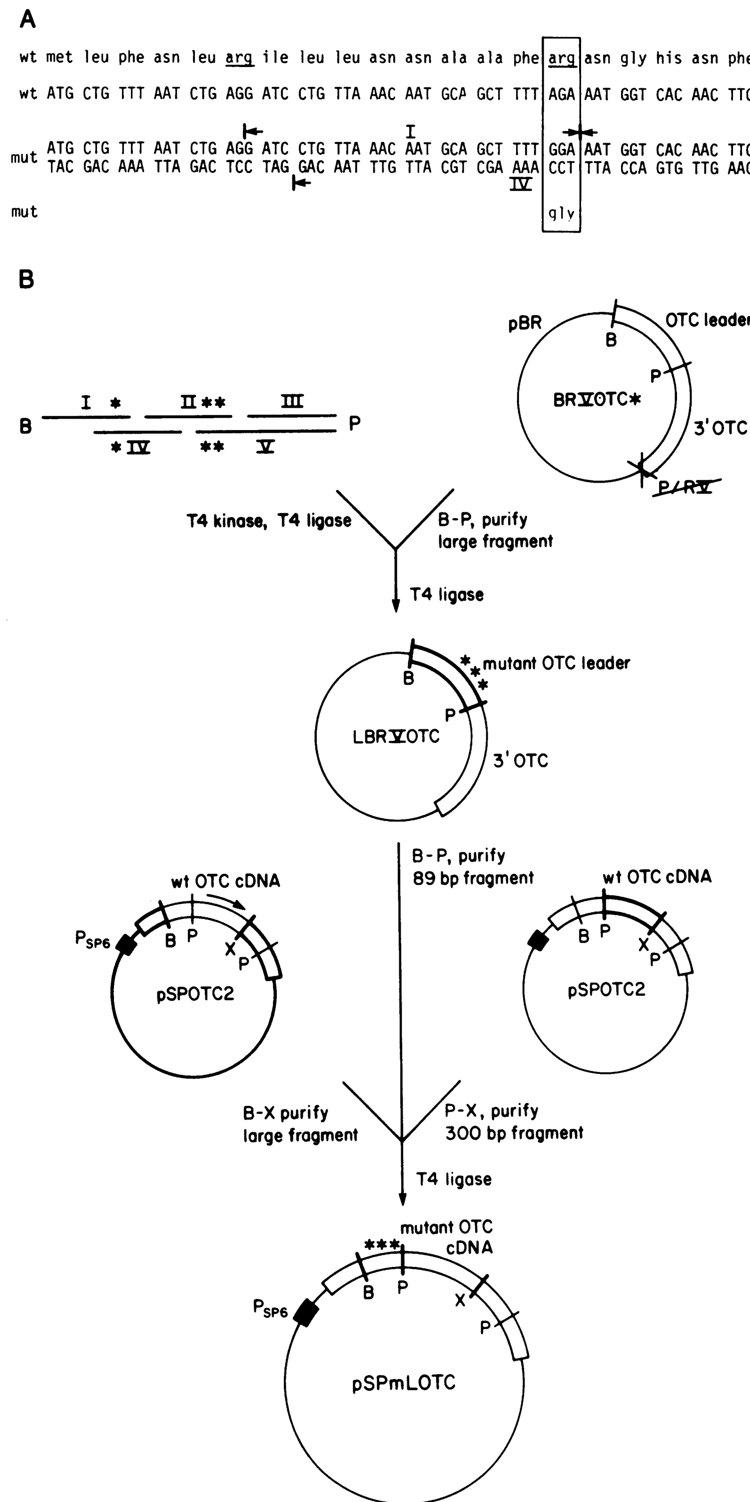

FIG. 1. Construction of plasmid containing a mutant (OTC) coding sequence joined with the prokaryotic promoter SP6. (*A*) Synthesis of a DNA coding fragment containing the desired mutations. The top two lines, designated wt, display the wild-type amino acid and nucleotide coding sequences. In the amino acid sequence, basic residues in the leader peptide are underlined and a vertical arrow designates the site of leader peptide cleavage. The bottom three lines, designated mut, display both strands of the mutant leader coding region and the residues substituted by mutation of the coding sequence. Five oligonucleotides were synthesized, designated I–V and delineated by horizontal arrows, composing an 89-base-pair (bp) *Bam*HI/*Pvu* II DNA fragment. The segment contains the codons for the distal 26 OTCase leader residues and 4 adjoining codons for the first four residues of the mature subunit. The segment differs from the wild-type sequence in the leader portion at codons 15, 23, and 26 (enclosed in boxes), where arginine has been changed to glycine. (*B*) Joining of oligonucleotides with each other and with remaining OTCase sequences to construct the mutant coding sequence downstream from an SP6 promoter. The five oligonucleotides containing mutations, designated by asterisks, were phosphorylated at their 5′ termini, joined together, and cloned as a *Bam*HI/*Pvu* II restriction fragment. This fragment, designated by thickened lines in the plasmid LBRVOTC, was joined with two additional fragments, similarly designated and independently excised from plasmid pSPOTC2, to produce the plasmid pSPmLOTC. The arrow next to the OTCase coding region of pSPOTC2 indicates the normal direction of transcription. Loss of *Eco*RV and *Pvu* II recognition sequences at a site of joining is indicated by ×. B, *Bam*HI; P, *Pvu* II; X, *Xho* I; pSP6, SP6 promoter sequence.

of precursors inside *Neurospora* mitochondria in the presence of *o*-phenanthroline, a chelator that inhibits the action of the Zn^{2+}-dependent matrix protease. Their observations, confirmed by us using the OTCase system (unpublished observations), clearly demonstrate that import and proteolytic cleavage are independent events. It therefore seems probable that we have observed here two independent effects of the glycine-substituted leader peptide—one on uptake and one on proteolytic cleavage.

The failure of uptake of the altered OTCase precursor most likely involves its lack of recognition by an outer membrane receptor similar to the one demonstrated for apocytochrome *c* of *Neurospora* (16). Analysis of the binding of OTCase precursors by isolated mitochondria should verify such a failure of recognition. The question arises as to how one or more of the arginine residues in the OTCase leader peptide contribute to its function in uptake. Three general possibilities can be mentioned. The first is that charge alone plays a role. In this event, the observed disruption of function could be simply explained as the result of conversion of one or more positively charged arginine residues to a charge-neutral species. A second possibility is that one or more of the arginine residues contributes to a specific amino acid sequence that mediates leader function. For example, nuclear localization of simian virus 40 T antigen has recently been shown to be directed by a specific sequence composed of six

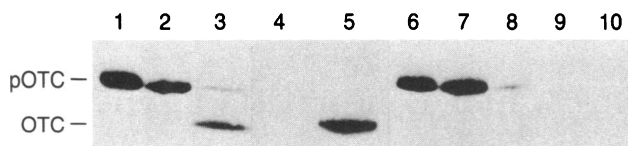

FIG. 2. Synthesis of wild-type and mutant OTCase precursors and incubation with isolated mitochondria. The plasmids pSPmLOTC and pSPOTC2 were transcribed *in vitro* and the products were used to direct reticulocyte lysate-programmed translation reactions containing [^{35}S]methionine. After translation, aliquots of the mixtures were immunoprecipitated with anti-OTCase antiserum. The remainder of the mixtures was incubated for 60 min at 27°C with isolated rat liver mitochondria. The mixtures were then divided: half of each was separated by centrifugation into supernatant and mitochondrial fractions; the remaining halves were first incubated with trypsin, and then also separated. The fractions were detergent-solubilized and immunoprecipitated with anti-OTCase antiserum. The products were electrophoresed through a $NaDodSO_4$/polyacrylamide gel, and the gel was fluorographed. Products synthesized from the wild-type plasmid pSPOTC2: directly immunoprecipitated (lane 1); supernatant after incubation with mitochondria (lane 2); mitochondrial pellet (lane 3); supernatant after trypsin treatment of mitochondrial incubation mixture (lane 4); mitochondrial pellet from trypsin-treated incubation mixture (lane 5). Products synthesized from the mutant plasmid pSPmLOTC: directly immunoprecipitated (lane 6); supernatant after incubation with mitochondria (lane 7); mitochondrial pellet (lane 8); supernatant after trypsin treatment of mitochondrial incubation mixture (lane 9); mitochondrial pellet from trypsin-treated incubation mixture (lane 10). pOTC, OTCase precursor (40 kDa); OTC, OTCase mature subunit (36 kDa).

consecutive residues, five of which are basic (3). A third possibility is that the arginine residues contribute to a secondary or tertiary structure, which is the functional element. We cannot at present distinguish among these possibilities, although we note, concerning the second possibility, that extensively shared amino acid sequences have not been identified in those leader peptides analyzed to date. A case in point is the OTCase leader peptide itself, whose sequence in rat has recently been determined and compared with that in man (17, 18). Ten amino acid differences were noted distributed throughout the sequence of 32 residues. Significantly, the four arginine residues present in the human leader peptide are precisely conserved in the rat peptide.

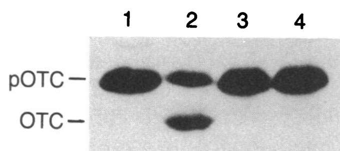

FIG. 3. Incubation of wild-type and mutant OTCase precursors with mitochondrial matrix fraction. Reticulocyte lysate containing either wild-type or mutant *in vitro*-synthesized precursor was incubated at 27°C for 1 hr with a mitochondrial matrix fraction, in the presence of 0.10 mM $ZnCl_2$. After incubation, the mixture was detergent-solubilized and immunoprecipitated with anti-OTCase antiserum. The products were electrophoresed through a $NaDodSO_4$/polyacrylamide gel, and the gel was fluorographed. Product synthesized from the wild-type plasmid pSPOTC2 (lane 1); after incubation with matrix fraction (lane 2). Product synthesized from the mutant plasmid pSPmLOTC (lane 3); after incubation with matrix fraction (lane 4). pOTC, OTCase precursor (40 kDa); OTC, OTCase mature subunit (36 kDa).

Role of Arginine in Proteolytic Cleavage. The failure of the altered precursor to be cleaved by the matrix fraction was unexpected. The nearest arginine residue lies eight residues from the site of proteolytic cleavage and, therefore, might not be expected to be involved with proteolysis. Several recent studies, however, have suggested that amino acids remote from the site of leader peptide cleavage may play a role in proteolysis. Hurt *et al.* (6) programmed synthesis of a chimeric precursor lacking the normal site of cleavage but observed proteolytic cleavage, nonetheless, at an upstream site. Horwich *et al.* (7) preserved the normal site of cleavage in a chimeric precursor, yet unexpectedly observed proteolysis at unusual positions. By analogy, one or more of the altered arginine residues could be members of a primary amino acid sequence normally recognized by the protease. It seems more likely, however, that the arginines contribute to a secondary or tertiary structure recognized by the matrix enzyme. It will be of considerable interest to determine which of the arginine residues altered in this study play a role in import and which play a role in proteolysis.

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1. Walter, P., Gilmore, R. & Blobel, G. (1984) *Cell* **38**, 5–8.
2. Kalderon, D., Roberts, B. L., Richardson, W. D. & Smith, A. E. (1984) *Cell* **39**, 499–509.
3. Hay, R., Bohni, P. & Gasser, S. (1984) *Biochim. Biophys. Acta* **779**, 65–87.
4. Maccecchini, M.-L., Rudin, Y., Blobel, G. & Schatz, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 343–347.
5. Van den Broeck, G., Timko, M. P., Kausch, A. P., Cashmore, A. R., Montau, M. V. & Herrera-Estrella, L. (1985) *Nature (London)* **313**, 358–363.
6. Hurt, E. C., Pessold-Hurt, B. & Schatz, G. (1984) *FEBS Lett.* **178**, 306–310.
7. Horwich, A. L., Kalousek, F., Mellman, I. & Rosenberg, L. E. (1985) *EMBO J.* **4**, 1129–1135.
8. Horwich, A. L., Fenton, W. A., Williams, K. R., Kalousek, F., Kraus, J. P., Doolittle, R. F., Konigsberg, W. & Rosenberg, L. E. (1984) *Science* **224**, 1068–1074.
9. Horwich, A. L., Fenton, W. A., Firgaira, F. A., Fox, J. E., Kolansky, D., Mellman, I. S. & Rosenberg, L. E. (1985) *J. Cell Biol.* **100**, 1515–1521.
10. Krieg, P. A. & Melton, D. A. (1984) *Nucleic Acids Res.* **12**, 7057–7070.
11. Conboy, J. G. & Rosenberg, L. E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3073–3077.
12. Conboy, J. G., Fenton, W. A. & Rosenberg, L. E. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1–7.
13. Fenton, W. A., Hack, A. M., Helfgott, D. & Rosenberg, L. E. (1984) *J. Biol. Chem.* **259**, 6616–6621.
14. Mori, M., Miura, S., Tatibana, M. & Cohen, P. P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7044–7048.
15. Zwizinski, C. & Neupert, W. (1983) *J. Biol. Chem.* **258**, 13340–13346.
16. Hennig, B., Koehler, H. & Neupert, W. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4963–4967.
17. Takiguchi, M., Miura, S., Mori, M., Tatibana, M., Nagata, S. & Kaziro, Y. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7412–7416.
18. Kraus, J., Hodges, P. E., Williamson, C. L., Horwich, A. L., Kalousek, F., Williams, K. R. & Rosenberg, L. E. (1985) *Nucleic Acids Res.* **13**, 943–952.