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Interaction of an Intermediate Structure of *Bacillus subtilis* α-Amylase With Alkyl-Substituted Sepharose 4B

A Model of Membrane Translocation

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Abstract

An intermediate form of α-amylase from *Bacillus subtilis* was prepared following a previously reported procedure. Stabilization of this protein structure by various additives and its interaction with alkyl-substituted Sepharose 4B (Sepharose-lipid), used to mimic the role of the alkyl chains of the phospholipid bilayer, were investigated. Exposure of hydrophobic clusters in the protein structure on denaturation was indicated by a greater affinity of the intermediate form for interaction with the alkyl chains on the matrix, as compared with the original native structure. Near- and far-ultraviolet circular dichroism studies supported loss of tertiary structure with retention of secondary structure, as expected from molten globular intermediate forms. Based on the results presented, we suggest that interaction of a protein in its native and nonnative forms with an alkyl-substituted matrix may provide useful information regarding its capacity for insertion into and translocation across the biologic membrane.

Index Entries: α-Amylase; intermediate structure; Sepharose-lipid; hydrophobic matrix; membrane insertion; membrane translocation.

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Introduction

After their synthesis in the cell, many proteins have to cross membranes in order to reach the site of their function. It has been established that this process is facilitated by partial unfolding of native protein molecules. Accordingly, to be competent for translocation across the cytoplasmic membrane, secretory proteins are *prevented* from folding into their native conformation in the cytosol of the cell. Following translocation, these proteins fold into their native conformation on the *trans* side of the membrane.

There is considerable evidence that in both prokaryotic and eukaryotic systems, the dynamics of protein unfolding-folding play an important role in secretion of exocellular proteins *(1–4)*. Furthermore, it has been suggested that in vitro studies of unfolding-folding transition of isolated exocellular proteins under in vivo conditions (such as pH and temperature) may provide useful information in relation to a better understanding of translocation processes in the cell *(4)*.

Bacillus subtilis and related *Bacillus* species are widely used in biotechnology because they are one of the best known prokaryotes. They are nonpathogenic, are capable of secreting functional extracellular proteins directly to the culture medium, and are currently used to produce various industrial enzymes. In a study on *B. subtilis* α-amylase, an intermediate protein structure was identified and suggested to be involved in the secretion process of this enzyme *(4)*. In the present study, the same denaturation strategy was employed, and stabilization of the intermediate structure by various stabilizers and its interaction with alkyl residues on hydrophobic adsorbents were investigated. We suggest that the type of study reported here may provide a useful model related to translocation of the enzyme.

Materials and Methods

Chemicals

α-Amylase from *B. subtilis* N7 was obtained from Merck (Darmstadt, Germany). α-Glucosidase was a product of Roche (Boehringer Mannheim), and Sepharose 4B and all other biochemicals were purchased from Sigma (St. Louis, MO). The amylase appeared homogeneous as judged by sodium dodecyl sulfate-gel electrophoresis run according to Laemmli *(5)*. Reproducibility of the data presented was confirmed by repeating the experiments at least twice.

Determination of Enzymatic Activity and Protein Concentration

α-Amylase was normally assayed at 37°C using blocked *p*-nitrophenyl α-D-maltoheptaoside as substrate and following a procedure essentially as described previously *(6)* with the difference that 100 m*M* potassium phosphate, pH 7.0, was used and α-glucosidase was added at 0.4 U/mL. Enzymatic activity was also measured by determining the concentration of reducing sugars on hydrolysis of soluble starch, as described previously *(7)*. Protein concentration was determined by the Bradford method *(8)*.

Preparation of Intermediate Form of Enzyme

Essentially, the procedure previously reported by Haddaoui et al. *(4)* for preparation of an intermediate form of the enzyme from a *B. subtilis* source was followed. A 7 mg/mL solution of α-amylase was extensively dialyzed in 100 m*M* potassium phosphate, pH 7.0. A volume of this enzyme solution was added to an appropriate volume of 6 *M* guanidinium hydrochloride (Gdn-HCl) prepared in the phosphate buffer containing 0.5 m*M* EDTA, providing a final mixture consisting of about 1.2 mg/mL of enzyme, 5*M* Gdn-HCl, and 0.5 m*M* EDTA. This preparation was incubated at 37°C for 10 min and subsequently diluted 60-fold in the phosphate buffer containing 0.5 m*M* EDTA at 37°C. The final preparation was left on ice until use.

Coupling of Alkyl Glycidyl Ethers to Sepharose 4B

Preparation of octyl-, dodecyl-, and palmityl-glycidyl ethers, their coupling to Sepharose 4B, and determination of the degree of substitution were carried out exactly as outlined previously *(9)*.

Preparation of Immobilized Protein

Adsorption of native α-amylase was tested by adding 1 mL of a 0.1 mg/mL solution of the enzyme (in the phosphate buffer) to 1 mL of Sepharose-lipid suspension containing 0.5 mL of the packed adsorbent at 25°C. The mixture was gently shaken for 60 min followed by centrifugation and washing of the pellet, twice with the same buffer. To immobilize the intermediate form (in the phosphate buffer containing 0.5 m*M* EDTA), the protein was concentrated fivefold by ultrafiltration at 4°C immediately after its preparation. One milliliter of the protein solution containing 0.1 mg of protein was added to 1 mL of the Sepharose-lipid suspension. The procedure described for the native enzyme was followed. On centrifugation, the pellets were washed with the phosphate buffer containing 0.5 m*M* $CaCl_2$. The activity of immobilized preparations was determined following a procedure reported previously *(10)*.

Circular Dichroism Measurement

Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with protein concentrations of about 0.2 and 1.0 mg/mL for far- and near-ultraviolet (UV) regions, respectively.

Fluorescence Measurements

Measurements were taken on a Hitachi MPF-4 apparatus. A 0.02 mg/mL solution of the enzyme was used for intrinsic fluorescence studies with an excitation wavelength of 280 nm. For extrinsic fluorescence measurements, the final 8-anilino-l-naphthalene sulfonate (ANS) and protein concentrations were 33 μ*M* and 0.02 mg/mL, respectively. An excitation wavelength of 350 nm was used.

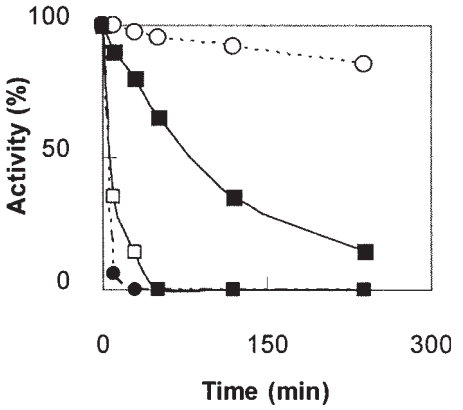

Fig. 1. Activity loss of intermediate structure (0.02 mg/mL) incubated in 100 m*M* phosphate buffer, 0.5 m*M* EDTA, pH 7.0 at (○) 4, (■) 25, (□) 37, and (●) 50 °C. Further details are described in Materials and Methods.

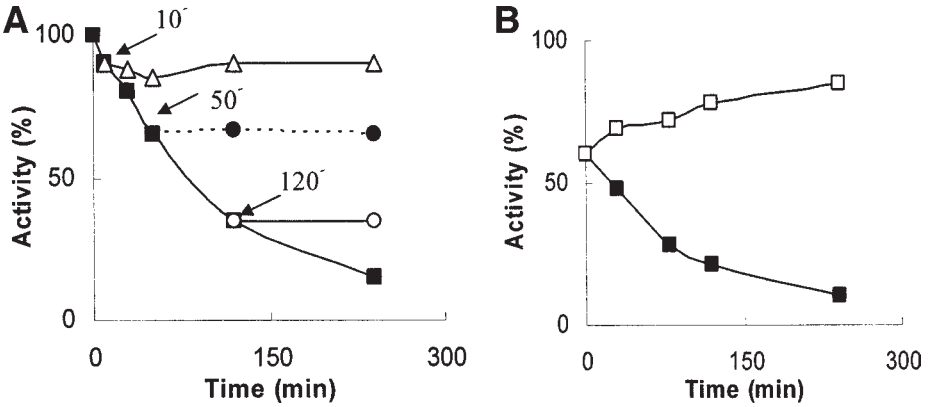

Fig. 2. Change in catalytic activity (at 25°C) with time of enzyme pretreated with 5 *M* Gdn-HCl and diluted in the buffer containing (■) 0.5 m*M* EDTA or (□) 0.5 m*M* $CaCl_2$. **(A)** Addition of $CaCl_2$ (0.5 m*M* final concentration) at 10 (△), 50 (●), and 120 (○) min to the intermediate structure. **(B)** The activity at zero time corresponding to the reading taken immediately after dilution and changes in activities with time in the two buffers are indicated. For additional details *see* Materials and Methods.

Results and Discussion

Formation of Intermediate Structure and Effect of Stabilizers

Immediately after preparation, the intermediate structure had 60% of the activity of the original native enzyme, was unstable, and lost its catalytic activity with rates depending on the temperature of incubation (4–50°C; see Fig. 1). This loss of activity was immediately and efficiently stopped by the addition of Ca^{+2}, and no further loss was observed when this inorganic ion was added to the preparation at different times of incubation at 25°C (Fig. 2A). In addition, when the denatured enzyme was diluted in the phosphate buffer containing 0.5 m*M* $CaCl_2$, instead of 0.5 m*M* EDTA,

Table 1
Adsorptive Immobilization of Native and Intermediate Forms of α-Amylase on Sepharose-Lipid[a]

Form of α-amylase	Immobilization (%)	Specific activity (U/mg)
Native		
C16	10 ± 3	24
Intermediate		
C8	30 ± 4	4
C12	40 ± 5	4.5
C16	52 ± 7	4

[a]Immobilization of native α-amylase was carried out on palmityl-Sepharose. For the intermediate structure, this process was carried out using octyl-, dodecyl-, and palmityl-Sepharose. For additional details, *see* Materials and Methods.

the intermediate structure regained most of its catalytic activity (Fig. 2B), presumably by going toward its native conformation, as also suggested by a similar study *(4)*. Calcium has been reported to exhibit the roles of allosteric activator *(11)* and protein stabilizer *(12)* in relation to α-amylases. All α-amylases contain at least 1 g atom of this inorganic ion/mol, with binding sites conserved in the enzyme isolated from different sources *(13)*.

Interaction of Native and Intermediate Forms of α-Amylase With Sepharose-Lipid

Native α-amylase showed a low affinity for adsorption, and only limited immobilization of the enzyme was observed using palmityl-Sepharose as the matrix (Table 1). Interaction of the enzyme with the hydrophobic supports was improved on conversion to the intermediate form. As indicated in Table 1, the protein showed a higher affinity for adsorption on palmityl-Sepharose than on the other two adsorbents, containing shorter alkyl chains, with comparable specific activities. These results suggest that by conversion of native α-amylase to an intermediate form, the hydrophobic pockets become more available for interaction with alkyl residues on the support. Similar conclusions were made in studies related to interaction of carbonic anhydrase *(14)*, urease *(15)*, and glucose oxidase *(16)* with hydrophobic supports.

Our previous studies related to interaction of proteins with Sepharose-lipid indicated that virtually irreversible binding may take place with some protein structures, even in their native forms *(11)*. In the present investigation, the adsorbents containing alkyl residues, such as palmityl groups, were used as models to mimic the hydrophobic properties of the phospholipid bilayer in relation to translocation of α-amylase, via formation of an intermediate form, as described previously *(4)*. Accordingly, the hydrophobic component of interactions between a protein molecule and phospholipids of biologic membranes is emphasized. For this model to be of

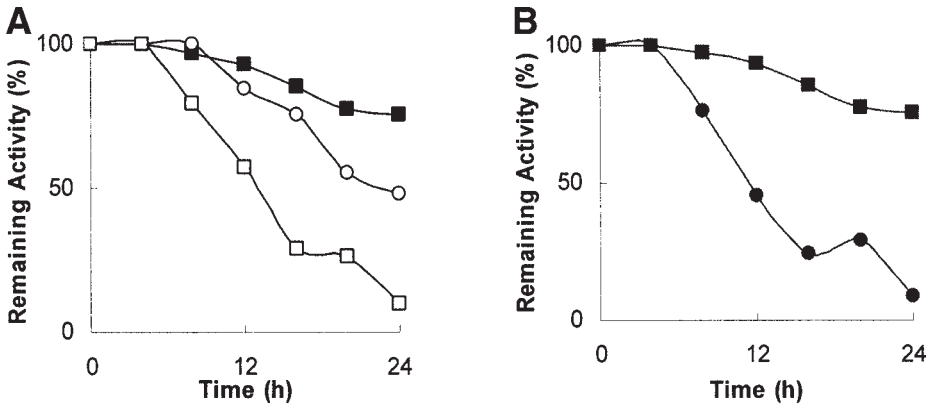

Fig. 3. Continuous catalytic operation involving immobilized preparations of intermediate form of α-amylase. Exactly the same amount of the intermediate form (100 μg) was loaded onto columns containing the hydrophobic adsorbents: **(A)** columns containing intermediate form of enzyme immobilized on (■) palmityl-, (○) dodecyl-, and (□) octyl-Sepharose; **(B)** columns containing intermediate form immobilized on palmityl-Sepharose and run at (●) 4 and (■) 25°C. Continuous hydrolysis of a 0.1% solution of soluble starch (prepared in 20 m*M* Tris, pH 7.0, containing 5 m*M* $CaCl_2$) was determined. Further details are described in Materials and Methods.

relevance to translocation, it would be expected that interaction of the intermediate structures with Sepharose-lipid takes place in the absence of strong associations. To test this, the immobilized preparations were used in continuous catalytic operations, which indicated ease of desorption in all cases (Fig. 3A). As expected, owing to relatively weaker hydrophobic interactions, the protein was more easily desorbed from octyl-Sepharose than from dodecyl- or palmityl-Sepharose (Fig. 3A). In addition, the degree of interaction was dependent on the temperature of operation (Fig. 3B).

The importance of this observation in relation to protein secretion is based on the premise that on conversion of native structures to intermediate forms, the hydrophobic residues exposed to the surface may induce membrane insertion and facilitate threading of the protein through the phospholipid bilayers, which would otherwise be difficult owing to high energy barriers. Accordingly, membrane insertion and translocation of this secretory protein becomes possible by its capacity to take up an intermediate form, as described in the present investigation. The information provided by this type of study could be useful in design strategies related to improvement of secretion of such proteins. An adsorbent containing palmityl residues may serve as a relatively more realistic model since palmityl groups constitute one of the most common acyl chains of the phospholipid molecules in biomembranes.

Secondary and Tertiary Protein Structures

Lowering of intrinsic fluorescence suggested displacement of tryptophan residues toward a relatively more polar environment, on formation

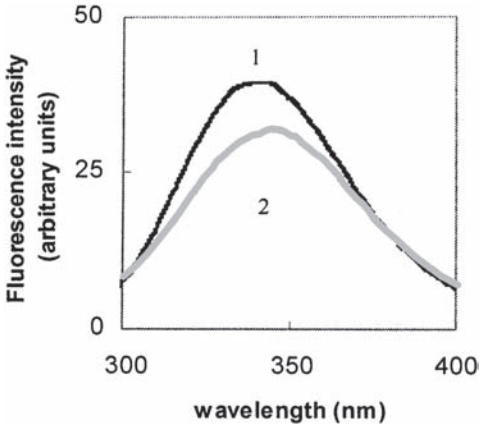

Fig. 4. Intrinsic fluorescence spectra of native (1) and intermediate (2) forms of α-amylase. Fluorescence spectra were taken 30 min after preparation of the intermediate structure. Further details are provided in Materials and Methods.

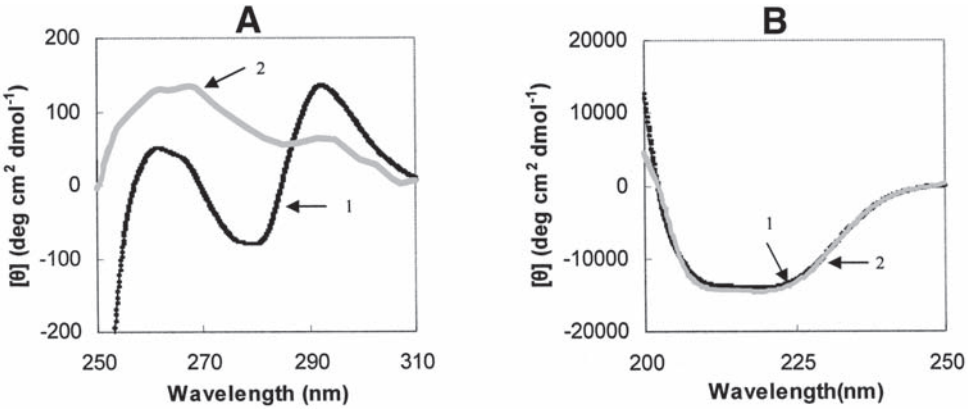

Fig. 5. **(A)** Near- and **(B)** far-UV spectra of native (1) and intermediate (2) forms of α-amylase. Additional details are provided in Materials and Methods.

of the intermediate structure (Fig. 4). In the near-UV CD spectra, clear differences in the tertiary structure of the native and intermediate forms are evident (Fig. 5A). The differences in the 260- to 270-nm and about 290-nm regions of the spectra may be taken to suggest that flexibilities of phenylalanine and tryptophan residues are diminished and enhanced, respectively, on formation of the intermediate form. The far-UV spectra (Fig. 5B) indicate that the secondary structure of the intermediate state is close to that of the corresponding native state. Taken together, these data suggest that the protein takes up an intermediate structure with some of the characteristics of molten globular forms *(17)*. The fact that the enzyme may take up such structures for its translocation may be of importance related to the mechanisms of secretion of this and other secretory proteins. The involvement of

the molten globular state of protein for protein translocation has already been suggested for other proteins (refs. *18* and *19*).

The aforementioned results suggest that the folding intermediate used in the present investigation displays different properties related to residual enzyme activity, stability, and CD characteristics as compared with those reported by Haddaoui et al. *(4)*. This is probably owing to the fact that the enzyme used in the present study was purified from *B. subtilis* N7, which has a molecular mass of 48 kDa, corresponding to a truncated form of the enzyme (molecular mass = 69 kDa) used by Haddaoui et al. *(4)*.

The results presented here on the enzyme gaining the capacity for interaction with the hydrophobic supports (Table 1) can obviously be explained in terms of the provision of hydrophobic sites on its denaturation. In some similar studies (*see* ref. *14*), we were able to demonstrate that this property of a protein may coincide with enhancement of the fluorescence of ANS, normally used as a hydrophobic-reporter probe. This dye has been shown to be a sensitive probe for partially folded intermediates (*see* refs. *20–22*). In the present investigation, however, we did not obtain a clear fluorescence enhancement of the probe, presumably owing to repulsive interactions between ANS and α-amylase (p*I* of 5.4). It is therefore suggested that had it not been for such repulsive electrostatic interactions or some other limitations *(23)*, the typical enhancement reported for similar protein structures (*see* ref. *24*) would have been attained.

The suggestion that the protein takes up some of the characteristics of molten globular intermediate structures may be of direct relevance to its ability to be translocated across biologic membranes. Various reports suggest that (*see* refs. *4*, *25*, and *26*) formation of such intermediate structures may indeed provide a protein with this capacity. This makes good sense because preservation of the secondary structure (in contrast to complete unfolding), concomitant with provision of hydrophobic sites (for interaction with alkyl groups of the phospholipids bilayer), may provide a protein with the best pathway for membrane insertion and translocation. Needless to say, the energy cost related to this process would be minimized with a compact architecture. An increase in the flexibility of parts of the protein structure on formation of the intermediate form, as suggested by the data presented in Fig. 5A, could also be of relevance because the protein should be sufficiently deformable for its translocation to occur efficiently.

It should be emphasized that conversion of native to nonnative α-amylase appears to provide the enzyme with *limited* capacity for interaction (as opposed to *extensive* "virtually irreversible" interaction; *[27]*). This is intriguing because there are reports in the literature (*see*, ref. *28*) suggesting that although an increase in the hydrophobicity of a signal peptide would improve protein secretion, there appears to be a limit to this effect and too high a hydrophobicity would actually reduce the secretion efficiency of proteins. To what extent the analogy between these two very dissimilar situations would be valid is open to conjecture. However, it is

clear that an efficient membrane translocation of a protein could only take place in the *absence* of extensive hydrophobic interactions.

Conclusion

We suggest that studies similar to the present investigation may provide information useful for a better understanding of some of the mechanisms related to membrane insertion and translocation of a protein. They may also furnish data useful for design strategies related to optimization of protein secretion.

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References

1 Eilers, M. and Schatz, G. (1988), *Cell* **52(4),** 481–483.
2 Simon, S. M., Peskin, C. S., and Oster, G. F. (1992), *Proc. Natl. Acad. Sci. USA* **89(9),** 3770–3774.
3 Song, T. and Park, C. (1995), *J. Mol. Biol.* **253(2),** 304–312.
4 Haddaoui, E., Leloup, L., Petit-Glatron, M. F., and Chambert, R. (1997), *Eur. J. Biochem.* **249,** 505–509.
5 Laemmli, U. K. (1970), *Nature* **227(259),** 680–685.
6 Hagele, E. O., Schaich, E., Rauscher, E., Lehmann, P., Burk, H., and Wahlefeld, A. W. (1982), *Clin. Chem.* **28(11),** 2201–2205.
7. Bernfeld, P. (1955), in *Methods in Enzymology*, vol. 1, Collowick, S. P. and Kaplan, N. O., eds., Academic, New York, pp. 149–158.
8 Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
9 Nemat-Gorgani, M. and Karimian, K. (1982), *Eur. J. Biochem.* **123(3),** 601–610.
10 Azari, F. and Nemat-Gorgani, M. (1999), *Biotechnol. Bioeng.* **62(2),** 193–199.
11 Feller, G., Payan, F., Theys, F., Qian, M., Haser, R., and Gerday, C. (1994), *Eur. J. Biochem.* **222(2),** 441–447.
12 Feller, G., d'Amico, D., and Gerday, C. (1999), *Biochemistry* **38(14),** 4613–4619.
13 Machius, M., Declerck, N., Huber, R., and Wiegand, G. (1998), *Structure* **6(3),** 281–292.
14 Hosseinkhani, S. and Nemat-Gorgani, M. (2003), *Enzyme Microb. Technol.* **33(2–3),** 179–184.
15 Azari, F., Hosseinkhani, S., and Nemat-Gorgani, M. (2001), *Appl. Biochem. Biotechnol.* **94(3),** 265–277.
16 Hosseinkhani, S., Moosavi-Movahedi, A. A., and Nemat-Gorgani, M. (2003), *Appl. Biochem. Biotechnol.* **110(3),** 165–174.
17 Christensen, H. and Pain, R. H. (1991), *Eur. Biophys. J.* **19(5),** 221–229.
18 Bychkova, V. E., Pain, R. H., and Ptitsyn, O. B. (1988), *FEBS Lett.* **238(2),** 231–234.
19 Schwartz, M. P., Huang, S., and Matouschek, A. (1999), *J. Biol. Chem.* **274(18),** 12,759–12,764.
20 Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas', A. F., and Gilmanshin, R. I. (1991), *Biopolymers* **31(1),** 119–128.
21 Muzammil, S., Kumar, Y., and Tayyab, S. (1999), *Eur. J. Biochem.* **266(1),** 26–32.
22 Koshiba, T., Yao, M., Kobashigawa, Y., Demura, M., Nakagawa, A., Tanaka, I., Kuwajima, K., and Nitta, K. (2000), *Biochemistry* **39(12),** 3248–3257.
23 Matulis, D., Baumann, C. G., Bloomfield, V. A., and Lourien, R. E. (1999), *Biopolymers* **49(6),** 451–458.

24. Rajaraman, K., Raman, B., and Rao, C. M. (1996), *J. Biol. Chem.* **271(44),** 27,595–27,600.
25 Shin, I., Kreimer, D., Silman, I., and Weiner, L. (1997), *Proc. Natl. Acad. Sci. USA* **94(7),** 2848–2852.
26 Van der Goot, F. G., Gonzalez-Manas, J. M., Lakey, J. H., and Pattus, F. (1991), *Nature* **354(6352),** 408–410.
27. Nemat-Gorgani, M. and Karimian, K. (1983), *Biotechnol. Bioeng.* **25,** 2617–2630.
28. Wong, S. L. (1995), *Curr. Opin. Biotechnol.* **6(5),** 517–522.