0273-2289/04/118/0011/$25.00

Reductive Alkylation of Lipase

Experimental and Molecular Modeling Approaches

Raja Noor Zaliha A. Rahman,*[1,2] Bimo Ario Tejo,[1] Mahiran Basri,[1,2] Mohd. Basyaruddin A. Rahman,[2] Farid Khan,[3] Sharifuddin M. Zain,[4] Teruna J. Siahaan,[5] and Abu Bakar Salleh[1,2]

[1]Enzyme and Microbial Technology Research, Faculty of Science and Environmental Studies, and [2]Laboratory of Enzyme and Microbial Technology, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Malaysia, E-mail: rnzaliha@fsas.upm.edu.my; [3]Centre for Protein Engineering, Department of Chemistry, Cambridge University, CB21EW Cambridge, UK; [4]Department of Chemistry, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia; and [5]Department of Pharmaceutical Chemistry, University of Kansas, 2095 Constant Avenue, Lawrence, KS 66047

Received March 25, 2003; Revised September 10, 2003; Accepted September 18, 2003

Abstract

Candida rugosa lipase was modified via reductive alkylation to increase its hydrophobicity to work better in organic solvents. The free amino group of lysines was alkylated using propionaldehyde with different degrees of modification obtained (49 and 86%). Far-ultraviolet circular dichroism (CD) spectroscopy of the lipase in aqueous solvent showed that such chemical modifications at the enzyme surface caused a loss in secondary and tertiary structure that is attributed to the enzyme unfolding. Using molecular modeling, we propose that in an aqueous environment the loss in protein structure of the modified lipase is owing to disruption of stabilizing salt bridges, particularly of surface lysines. Indeed, molecular modeling and simulation of a salt bridge formed by Lys-75 to Asp-79, in a nonpolar environment, suggests the adoption of a more flexible alkylated lysine that may explain higher lipase activity in organic solvents on alkylation.

Index Entries: Lipase; *Candida rugosa*; enzyme modification; circular dichroism; molecular modeling.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Enzymes in organic solvents have been largely studied and employed in the areas of synthesis, food, and analysis *(1)*. Although enzymes in organic media show numerous advantages, their catalytic activity is in most cases orders of magnitude lower than in an aqueous environment *(2)*. This behavior can be ascribed to different causes: one of the reasons for this low catalytic activity is attributed to the hydrophobic/hydrophilic balance on the protein surface *(3)*.

To improve protein biocatalyst performance, protein surface characteristics could be altered. Chemical modification is a good strategy to improve enzyme performance in organic solvents *(4)*. Incrementing enzyme hydrophobicity through attachment of a hydrophobic group using aldehydes to the enzyme surface has been considered to increase enzyme solubility in a hydrophobic environment *(5)*.

Reductive alkylation is a convenient method to convert surface-exposed amino groups in proteins into their alkylamino derivatives. Unlike other procedures for the modification of amino groups of proteins, reductive alkylation has little effect on the physicochemical properties of protein *(5)*. Alkyl substitution of hydrogen will increase both the bulkiness and hydrophobicity of the amino groups and reduce the ability of amino groups to form hydrogen bonds *(3,6)*.

One of the frequently studied enzymes for increasing their activity in organic solvents through chemical modification is *Candida rugosa* lipase (EC 3.1.1.3). Its crystal structure has been resolved at 2.1-Å resolution *(7)*. It is a 57-kDa α/β protein (19 α- and 16 β-content) with an overall α/β-hydrolases core fold and 534 amino acid residues. This lipase has been frequently studied owing to its high activity in hydrolysis as well as in synthesis of lipids. In particular, it has been used for the kinetic resolution of racemic carboxylic acids and alcohol *(8)*. Furthermore, *C. rugosa* lipase has been chemically modified to enhance its activity in organic solvents. Salleh et al. *(9)* have attached a variety of hydrophobic groups to enzymes with different chain lengths via reductive alkylation of aldehydes. The thermostability of native and alkylated lipase was also investigated at various temperatures. Enzymes derivatized with longer alkyl groups (octyl and dodecyl) were slightly more stable than those modified with shorter alkyl group *(10)*. The effect of reductive alkylation of *Candida rugosa* lipase on its selectivity has been examined. The alkylated enzyme showed higher enantioselectivity toward esterification of (*R*)-2-(4-chlorophenoxy) propionic acid with *n*-tetradecanol *(11)*. However, the stabilization mechanisms of alkylated lipase in organic solvents have not been studied yet.

Our main goal in the present work was to examine further, at the molecular level, the mechanisms of how alkylation can affect the enzyme structurally. We used two approaches: experimental spectroscopic observation and theoretical molecular dynamics simulation. In the experimental part, circular dichroism (CD) was used to observe such a change on the

enzyme structure, and the molecular modeling part showed how alkylation affects the particular lysine and how it interacts with neighboring residues. The findings presented here could give some insight into how alkylated proteins are stabilized in organic solvents and may help protein engineers to design modified enzymes that will work better in an anhydrous environment.

Materials and Methods

Purification of Enzyme

Crude preparation of *C. rugosa* lipase type VII (Sigma, St. Louis, MO) was used. The enzyme was purified using a modified method of Rua et al. *(12)*. Crude preparation was obtained using water extraction and concentrated using ethanol precipitation. Separation of isoforms was achieved at 4°C using a DEAE-Sephacel (Amersham Pharmacia, Piscataway, NJ) column (2 × 28 cm) equilibrated with 25 m*M* Tris-HCl (Merck KgaA, Darmstadt, Germany) buffer and eluted with a 0–0.4*M* NaCl gradient in the same buffer with a flow rate of 1 mL/min. Two peaks with lipase activity were obtained, isolipase A and B, respectively. Isolipase B was loaded on a Sephadex G-100 (Sigma) column (2×100 cm) equilibrated with distilled water. Lipase-containing fractions were pooled, concentrated, and freeze-dried.

Reductive Alkylation

Modification of isolipase B with propionaldehyde was carried out in 0.1*M* borate buffer (Merck KgaA), pH 9.0, at 4°C. Propionaldehyde (Fluka) was added repeatedly with different molar ratios to protein to obtain different degrees of modification. Reduction of Schiff base formed to a stable secondary amine that was achieved by adding 4 to 5 mg of TNBS (Sigma). The solution was adjusted to pH 7.0 with 0.1*M* HCl and dialyzed against distilled water for 48 h. It was then lyophilized and kept at –20°C prior to use. The percentage of lysine modified was determined using the TNBS method according to Habeeb *(13)*.

Far-Ultraviolet CD Spectroscopy

Native and modified lipases (8 μ*m*) were measured in a Jasco 720 CD spectropolarimeter, and each sample was in 25 m*M* phosphate buffer (pH 7.5). Spectra were measured at 37°C using a 0.2-cm path length, 50-mdeg sensitivity, and 0.5-nm step resolution.

Analysis of CD Spectra

CD spectra were used to analyze the change in secondary structures on alkylation. The values of molar residue ellipticity were extracted from the spectra and used as numerical input for subsequent analyses. The secondary structure elements of native and alkylated lipases were analyzed using CDPro software *(14)*, provided by Dr. Narasimha Sreerama of Colo-

rado State University, and K2D *(15)* analysis software, provided by Dr. Miguel Andrade of EMBL Heidelberg. CDPro consists of CELCON3 (variable selection–self-consistent), CONTINLL (ridge regression), and CDSSTR (variable selection–random choice). K2D is a neural network–based program.

Molecular Modeling in Aqueous and Organic Solvent Models

Simulation of the modified lysines was performed using computer-aided molecular modeling on a Silicon Graphics O2 workstation. Starting Cartesian coordinates of closed lipase were obtained from the crystal structure (1TRH, Protein Data Bank). CVFF force field was used as the default option in the DISCOVER software (Insight II; Accelrys). Energy minimization and molecular dynamics were performed in a distant-dependent continuum solvent model using 80 (water) and 1 (nonpolar environment) as the dielectric constant (ε). Solvent-accessible surface area (SASA) analysis was performed using a Gauss–Bonnet algorithm implemented in GETAREA 1.1 software (www.scsb.utmb.edu/getarea) to determine the rank of solvent-exposed lysines that were possible to be alkylated.

Molecular dynamics and trajectories analysis of alkylated lysine were done using HyperChem software (Hypercube) with the AMBER96 force field. Alkylated lysine and its countercharged residue were heated to 300 K for 20 ps with a time step of 1 fs. The equilibration and production phases were then run for 80 ps. Other residues were restrained during simulation to save computing time.

Results and Discussion

Modification of Lipase

Isolipase B was chosen to be modified because its crystal structure has been resolved *(7)*. Two different degrees of modification were obtained (49 and 86%, respectively) with varying of the molar ratio of the modifiers to the protein.

Circular Dichroism

The far-ultraviolet (UV) CD spectrum of native isolipase B in aqueous buffer shows a broad minimum between 208 and 225 nm that is characteristic for an α/β class of protein *(16)*. However, there was a reduction in the CD spectra minima of the modified lipase with respect to the native enzyme, which is attributed to loss in both secondary and tertiary structure (Fig. 1). Furthermore, increasing the degree of modification from 49 to 86% resulted in further loss of structure. The secondary structure elements of the CD spectra of native and modified lipase were analyzed using the CDPro software and the K2D program. These different methods yield the same results in that the ratio of α-helical structure is lost (Table 1). In addition, the loss

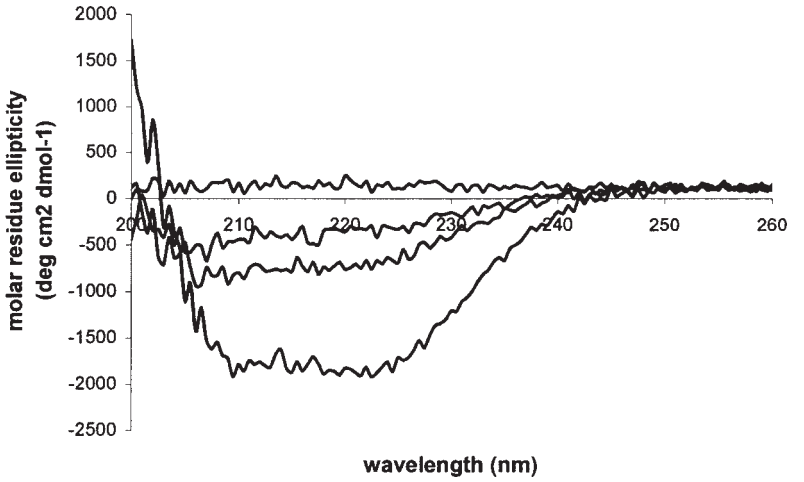

Fig. 1. Far-UV CD spectra of unmodified lipase, 49% lysines modified, 86% lysines modified, and buffer (from bottom to top, respectively).

in secondary structure of the modified lipase in aqueous buffer explains the reduced hydrolytic activity of alkylated lipase *(10)*.

Spectroscopic analysis for modified lipases in organic solvent was not performed. Analysis of secondary structure of protein in organic solvents using spectroscopic methods encountered some limitations related to protein solubility. Note that CD and fluorescence observations cannot be carried out in several solvents that are highly hydrophobic. In an observation of polyethylene glycol-complexed lipase, Secundo and Carrea *(17)* found that organic solvents more hydrophobic than dioxane cannot be used for spectroscopic analysis owing to a solubility problem.

Molecular Modeling in an Aqueous Solvent Model

Lys-75 was chosen as a model because of its position in the lid region that was fully exposed based on SASA calculations of the open form of *C. rugosa* lipase (Fig. 2). In this structure, residue Lys-75 forms a 3.6-Å salt bridge between the ε-amino group and the carbonyl group of Asp-79. After modification, the ε-amino of Lys-75 is 4.6 Å distant from Asp 79. This indicates that the latter position is less favorable in the formation of a salt bridge with respect to the native unmodified enzyme.

Of the 20 lysine residues present in *C. rugosa* lipase, 16 of these have salt bridges with its countercharged residues (data not shown). Indeed, salt bridges are an important factor for maintaining α-helix stability *(18)*. In particular, most of the ion pairs that obey the geometric definition of a salt bridge, namely, 4.0-Å distance between the charged group centroids and that which contains at least one pair of side-chain nitrogens and an oxygen atom within 4.0 Å, are stabilizing the proteins. On the other hand, those with a distance exceeding the 4.0-Å limit largely contribute to destabiliza-

Table 1
Secondary Structure Ratio of Modified and Unmodified Lipases Calculated Using Different Methods[a]

	SELCON3			CONTINLL			CDSSTR			K2D		
	U	M1	M2	U	M1	M2	U	M1	M2	U	M1	M2
H(r)	0.586	0.406	—	0.794	0.407	0.180	0.478	0.411	0.000	0.59	0.08	0.07
H(d)	0.224	0.190	—	0.201	0.183	0.194	0.149	0.119	0.000	—	—	—
S(r)	0.002	0.022	—	0.000	0.054	0.120	0.086	0.087	0.000	0.24	0.24	0.45
S(d)	0.001	0.026	—	0.005	0.028	0.040	0.080	0.084	0.000	—	—	—
Trn	0.045	0.114	—	0.000	0.086	0.070	0.030	0.074	0.000	0.07	0.51	0.48
Unrd	0.149	0.250	—	0.000	0.243	0.397	0.171	0.222	0.000	—	—	—

[a]U, unmodified lipase; M1, 49% modified; M2, 86% modified; H(r), regular α-helix; H(d), distorted α-helix; S(r), regular β-strand; S(d), distorted β-strand; Trn, turn; Unrd, unordered.

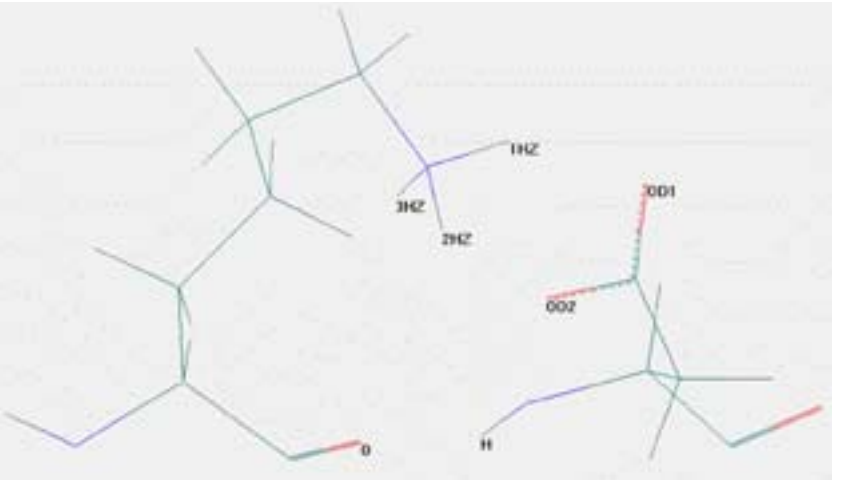

Fig. 2. Lys-75 (*left*) and Asp-79 (*right*) interacted through a salt bridge and hydrogen bonds.

tion of protein structure *(19)*. This might explain the loss of helical structure as shown by the CD spectra (Fig. 1). A series of simulations involving the whole protein molecule soaked in an explicit solvent are in progress.

It is clear from the CD spectra that at least in an aqueous environment the modified enzyme is unfolded. This makes sense in that helix formation requires burial of hydrophobic side chains *(20)*, and the introduction of alkylated groups on otherwise surface-exposed lysines would thermodynamically favor their burial and thus unfolding the lipase. However, the reduction in enzyme activity in an aqueous environment may not be the same as that of the modified lipase in organic solvent. In some cases, enzyme catalytic activity is enhanced in organic solvents *(21)*. Whereas alkylated surface-modified lysines of lipase may be thermodynamically unstable in aqueous buffer, in organic solvents, such groups may be stabilized through hydrophobic interactions while hydrophilic residues may be excluded. Furthermore, since the catalytic sites are buried in aqueous environments, organic solvents may expose such buried sites, which can enhance its activity.

Molecular Modeling in an Organic Solvent Model

The salt bridge of Lys-75–Asp-79 in organic solvent had a shorter distance of 2.7 and 3.5 Å in both the native and alkylated lysine lipase, respectively, compared with the enzyme in an aqueous system. Deviation of the simulated structures from its starting conformation was monitored by the root mean square deviation (RMSD) as a function of time. Based on RMSD plot, alkylated lysine had a more distant resemblance to the starting structure compared with unmodified lysine. It was deviated 1–1.5 Å from its initial structure at the equilibrium state. Unmodified lysine had a closer

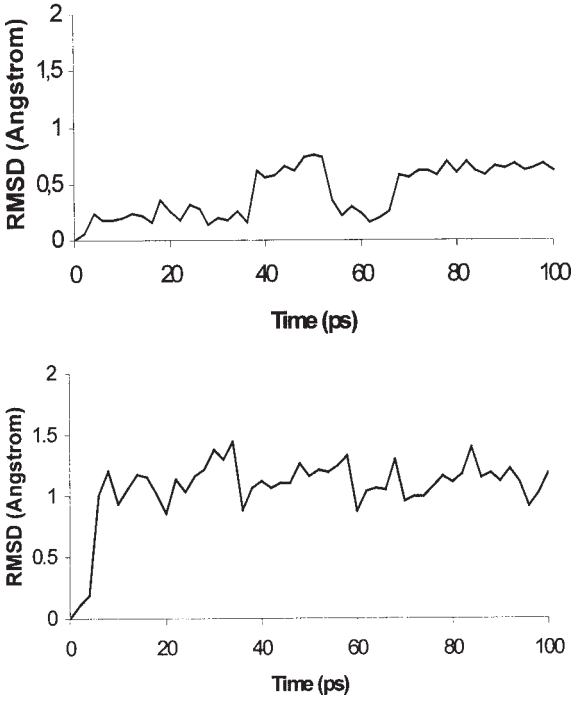

Fig. 3. RMSD fluctuation of unmodified **(A)** and modified **(B)** Lys-75 in a hydrophobic environment model (ε = 1).

resemblance to the initial structure with RMSD around 0.5 Å at the equilibrium state (Fig. 3). Larger deviations of alkylated lysine indicate that alkylation led the lysine to be more flexible than its unmodified form.

Attachment of alkyl chain on the lysine decreases the possibility of creating intramolecular interactions between lysine and its neighboring residues. Table 2 shows that alkylated lysine has fewer hydrogen bonds than unmodified lysine during 100 ps of molecular dynamics simulation. With fewer hydrogen bonds formed, enzyme would be more flexible, which is advantageous in increasing its catalytic activity in organic solvent.

Basically, enzyme would have more intramolecular interactions in organic solvents than in aqueous solvents. The higher number of intramolecular contacts can also be considered an important factor in determining the lower activity of enzymes in nonaqueous media: if the enzyme is, in fact, more rigid owing to intraprotein interactions, it will be less able to adapt to the substrates entering the active-site pocket *(22)* since binding-site selection is ligand dependent *(23)*.

Alkylation can decrease the intramolecular interactions, thus causing such an increment in flexibility of the enzyme. The full molecular dynamics simulations of alkylated lipase in CCl_4 solvent show that alkylated lipase has a lower number of hydrogen bonds not only at the particular lysines that are modified, but also at the amino acid residues that are not alkylated (data not shown).

Table 2
Hydrogen Bonds Formed Between Lys-75 and Asp-79
During 100-ps Molecular Dynamics

Time (ps)	Unmodified	Modified
0	H9-O4	H9-O4
2	H9-O4	H9-O4
4	—	H9-O4
6	2HZ-OD2/H9-O4	—
8	—	3HZ-OD1
10	2HZ-OD2	3HZ-OD1/H9-O4
12	2HZ-O4	3HZ-OD1
14	2HZ-OD2/H9-O4	3HZ-OD1/H9-O4
16	H9-O4	3HZ-OD1/H9-O4
18	—	—
20	2HZ-OD2/H9-O4	—
22	2HZ-O4	—
24	H9-O4	3HZ-OD1
26	2HZ-OD2	H9-O4
28	H9-O4	3HZ-OD1/H9-O4
30	H9-O4	—
32	H9-O4	3HZ-OD1
34	2HZ-OD2/H9-O4	3HZ-OD1/H9-O4
36	H9-O4	3HZ-OD1
38	1HZ-OD2 /H9-O4	3HZ-OD1/H9-O4
40	3HZ-O4 /H9-O4	3HZ-OD1/H9-O4
42	2HZ-OD1/3HZ-O4	—
44	3HZ-OD2/H9-O4	—
46	H9-O4	—
48	—	H9-O4
50	3HZ-OD1	H9-O4
52	—	H9-O4
54	2HZ-OD2/H9-O4	3HZ-OD1
56	2HZ-OD2	3HZ-OD1/H9-O4
58	2HZ-O4/H9-O4	3HZ-OD1
60	2HZ-O4/H9-O4	3HZ-OD1/H9-O4
62	1HZ-OD1	H9-O4
64	2HZ-OD2	H9-O4
66	H9-O4	H9-O4
68	1HZ-OD2	3HZ-OD1/H9-O4
70	1HZ-OD2/H9-O4	H9-O4
72	H9-O4	H9-O4
74	H9-O4	—
76	—	H9-O4
78	H9-O4	3HZ-OD1
80	1HZ-OD2	—
82	1HZ-OD2/H9-O4	3HZ-OD1/H9-O4
84	—	H9-O4
86	1HZ-OD2/H9-O4	3HZ-OD1/H9-O4
88	—	3HZ-OD1/H9-O4
90	H9-O4	—
92	—	3HZ-OD1/H9-O4
94	1HZ-OD2/H9-O4	H9-O4
96	—	3HZ-OD1/H9-O4
98	3HZ-OD1	H9-O4
100	H9-O4	H9-O4

Conclusion

In this initial study, some important insights were obtained on how alkylation could reduce the hydrolytic activity of lipase in an aqueous system and, on the other hand, could increase the synthetic activity in a hydrophobic environment. One of the critical points during transferring of enzymes from an aqueous environment to the hydrophobic milieu is related to loss of flexibility caused by the increment of intramolecular interactions. It was shown, at the molecular level, that alkylation could increase the flexibility of modified lysine and, at the same time, could decrease the possibility of enzyme forming intramolecular interactions.

Acknowledgments

We thank Prof. Nor Muhammad Mahadi (Biotechnology Academy, Universiti Kebangsaan Malaysia) for providing the computer-modeling facility and Priv. Doz. Dr. Jürgen Pleiss (Institut für Technische Biochemie, Universität Stuttgart) for enlightening discussions. This work was funded by the Malaysian Ministry of Science, Technology and Environment (grant no. 09-02-04-001 BTK/TD/004).

References

1. Koskinen, A. M. P. and Klibanov, A. M. (1996), *Enzymatic Reactions in Organic Media*, Blackie, London.
2. Klibanov, A. M. (1997), *Trends Biochem. Sci*. **15,** 97–101.
3. Ampon, K., Salleh, A. B., Basri, M., Yunus, W. M. Z., Razak, C. N. A., and Whitaker, J. R. (1993), *J. Biosci*. **4(2),** 154–160.
4. Longo, M. A. and Combes, D. (1999), *J. Chem. Technol. Biotechnol.* **74,** 25–32.
5. Inada, Y., Furukawa, M., Sasaki, H., Kodera, Y., Hiroto, M., Nishimura, H., and Matsushima, A. (1986), *TIBTECH* **13,** 86–91.
6. Means, G. E. (1977), in *Methods in Enzymology*, vol. XLVII, Hirs, C. H. W. and Timasheff, S. N., eds., Academic, New York, pp. 469–478.
7. Grochulski, P., Li, Y., Schrag, J. D., and Cygler, M. (1994), *Protein Sci*. **3,** 82–91.
8. Wu, S. H., Guo, Z. W., and Sih, C. J. (1990), *J. Am. Chem. Soc*. **112,** 1990–1995.
9. Salleh, A. B., Ampon, K., Salam, F., Wan Yunus, W. M. Z., Razak, C. N. A., and Basri, M. (1990), *Ann. NY Acad. Sci.* **613,** 521, 522.
10. Ampon, K., Salleh, A. B., Salam, F., Wan Yunus, W. M. Z., Razak, C. N. A., and Basri, M. (1991), *Enzyme Microb. Technol*. **13,** 597–601.
11. Basri, M., Th'ng, B. L., Razak, C. N. A., and Salleh, A. B. (1998), *Ann. NY Acad. Sci*. **864,** 192–197.
12. Rua, M. L., Diaz-Maurino, T., Fernandez, V. M., Otero, C., and Ballesteros, A. (1993), *Biochim. Biophys. Acta* **1156,** 181–189.
13. Habeeb, A. F. S. A. (1966), *Anal. Biochem*. **14,** 328–336.
14. Sreerama, N. and Woody, R. W. (2000), *Anal. Biochem*. **282,** 252–260.
15. Andrade, M. A., Chacon, P., Merelo, J. J., and Moran, F. (1993), *Protein Eng*. **6,** 383–390.
16. Manavalan, P. and Johnson, W. C., Jr. (1983), *Nature* **305,** 831–832.
17. Secundo, F. and Carrea, G. (2002), *J. Mol. Catal. B* **19–20,** 93–102.
18. Altamirano, M. M., Blackburn, J. M., Aguayo, C., and Fersht, A. R. (2000), *Nature* **403,** 617–622.
19. Kumar, S., Wolfson, H., and Nussinov, R. (2001), *IBM J. Res. Dev.* **45(3/4),** 499–512.
20. Richardson, J. S. and Richardson, D. C. (1988), *Science* **240,** 1648–1652.
21. Guo, Y. and Clark, D. S. (2001), *Biochim. Biophys. Acta* **1546,** 406–411.
22. Colombo, G., Ottolina, G., and Carrea, G. (2000), *Monat. Chem*. **131,** 527–547.
23. Ma, B., Wolfson, H., and Nussinov, R. (2001), *Curr. Opin. Struct. Biol*. **11,** 364–369.