BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 207–212 (1998)
ARTICLE NO. RC989107

Structural and Functional Role of Tryptophan in Xylanase from an Extremophilic *Bacillus:* Assessment of the Active Site

Devyani Nath and Mala Rao[1]
Division of Biochemical Sciences, National Chemical Laboratory, Pashan, Pune-411008 India

Received June 25, 1998

Microenvironment and conformation of the active site of xylanase from an extremophilic *Bacillus* was deciphered for the first time using fluorescence spectroscopy. NBS modified enzyme showed complete inactivation and the kinetic analysis implicated the presence of an essential tryptophan at the active site of xylanase. Xylan (0.5%) protected the enzyme completely from inactivation with NBS, whereas it afforded 35% protection against the loss of fluorescence, suggesting that not all the tryptophans are involved at the substrate binding site. Quenching studies revealed that acrylamide was more efficient than KI and CsCl as indicated by the higher Stern–Volmer quenching constants (Ksv). The steric factor represented by the percentage accessibility of the tryptophan residues of XylII was higher with the positively charged Cs^+ (80) than with the negatively charged I^- (10), suggesting that the tryptophan residues are located in a relatively electronegative environment. In the presence of 6 M Gdn HCl the fluorescence shifted to 350 nm with increased accessibility of the fluorophore to the quenchers. The proximity of the essential carboxyl groups with a high pKa value of 6.9 [Chauthaiwale and Rao (1994) *Biochim. Biophys. Acta*] probably contributes to the electronegative environment of the tryptophan residue. Our results on sequence analysis of the gene encoding for XylII (Accession Number U83602 in the GenBank database) have shown that Trp 61 is highly conserved and may play a role in the structure–function relationship of the enzyme. © 1998 Academic Press

Recently xylanases from extremophiles are gaining importance due to their biotechnological applications and as useful model systems for structure-function studies. Xylanases (EC.3.2.1.8) act on β-1,4 linked xylopyranosyl residues of the xylan backbone and in conjunction with cellulases convert the cellulosic biomass to sugars. Currently the most promising application for cellulase free xylanases has been reported in the paper and pulp industry [1]. Chemical modification studies are important in structure function analysis and protein engineering. The studies on sequence similarities of xylanases, cellulase and lysozyme have supported the possibility that catalytic mechanism of these enzymes follows the same pathway. The involvement of carboxyl groups for the catalysis [2] and the participation of tryptophan residues for the substrate binding [3] have been shown. Crystallographic analysis of the active site residues of xylanases of family G have revealed the presence of a large number of aromatic residues mainly tyrosines and tryptophans which comprise the binding or active sites [4]. Quenching reactions can provide information on the interactions with substrates or inhibitors and about intramolecular distances between specific chromophoric groups.

Extremophilic *Bacillus* secretes a low molecular weight xylanase (Xyl II) Mr 15,800 with a pI of 8 and belongs to the family G/11 according to the numerical classification of glycosyl hydrolases [5]. The enzyme exhibited stability and activity over a wide pH range of 7-10 at 60-70°C [6]. The applicability of this cellulase free thermostable xylanase for enzymatic bleaching has also been demonstrated by us [7]. Gene fragment encoding for the Xyl II has been cloned and expressed in *E. coli* [8] and *Bacillus* [9]. Nucleotide sequence of Xyl II has been carried out (manuscript under preparation) and site directed mutagenesis experiments to identify the active site residues is in progress. Despite the importance of extremophilic xylanases in biotechnological application no reports are documented deciphering the active sites and their microenvironment. In the present investigation we report the fluorometric interactions of extremophilic xylanase with the chemical modifier (NBS) and to probe the microenvironment

[1] To whom correspondence should be addressed. Fax: 0212-330233/0212-334761.

0006-291X/98 $25.00

of the tryptophan residues in the native and denatured form using solute quenchers.

MATERIALS AND METHODS

Production and purification of xylanase. Xylanase was produced and purified according to the earlier report [6]. Xylanase activity and protein was determined according to [10,11].

Reaction of xylanase with chemical modifier. The enzyme solution containing 0.2μg protein in buffer (50mM acetate buffer, pH 6.0) was incubated in the presence of various amounts of modifier for different time periods in a volume of 0.5ml. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions. The pseudo-first order rate constants (K) were obtained from the best fit plots of logarithm of the residual activity against time of reaction. The second order rate constants were calculated from the slopes of the plots of the pseudo-first order rate constants against concentration of the inhibitor [12]. Xylanase (1×10^{-5}M) in 50mM acetate buffer pH 6.0 was titrated with 10μl aliquots of NBS (1×10^{-4}M) and the number of tryptophan residues oxidized per mol of enzyme was determined spectrophotometrically according to Spande et al [13].

Fluorescence measurements and quenching experiments. All fluorescence studies were measured on an Perkin-Elmer Luminescence spectrometer LS 50B at 25°C. The results of the quenching reactions were analysed according to the Stern-Volmer and modified Stern-Volmer equation [14,15].

RESULTS

Tryptophanyl Fluorescence Analysis of Native and NBS Modified Xylanase

The native and NBS modified Xyl II fluoresce with an emission maximum of 339nm on excitation at 295nm (Fig. 1). The fluorescence spectrum of native enzyme showed a rapid decrease on addition of NBS upto a molar ratio of ~5. A negligible change on further addition was observed suggesting that in a molecule of Xyl II the

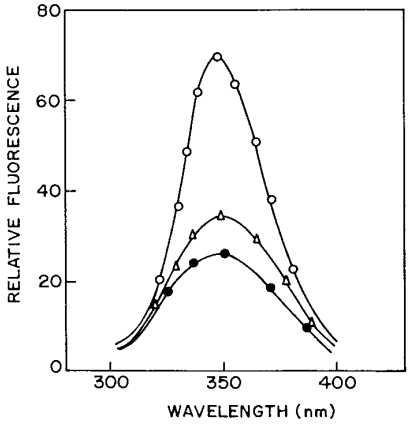

FIG. 1. Fluorescence spectra of 25 μg xylanase (○) treated with 20 μM NBS in presence of 0.5% xylan (△) and in presence of NBS alone (●).

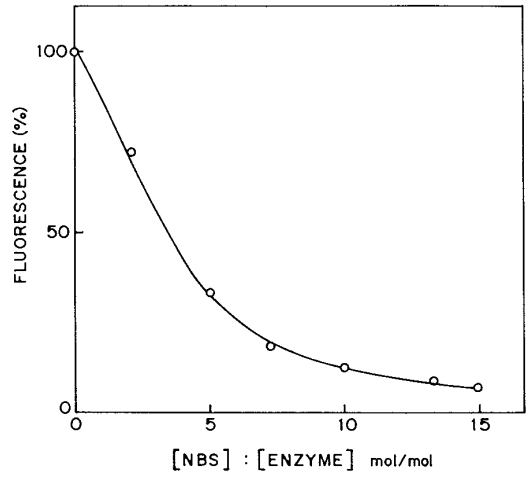

FIG. 2. Effect of NBS on the tryptophanyl fluorescence of xylanase. Enzyme (1.87×10^{-6}M) in 0.05 M sodium phosphate buffer pH 6.0 was titrated with aliquots of NBS (1×10^{-3}M).

reactivities of the tryptophans were different (Fig. 2). The tryptophanyl fluorescence analysis showed that the substrate xylan (0.5%) quenched the fluorescence to 50%. However, on addition of NBS a significant change in quenching of fluorescence was observed (Fig. 1).

Inactivation Kinetics of NBS Modified Enzyme

The number of tryptophan residues in Xyl II was found to be three from the method of Goodwin and Morton [16]. The inactivation of Xyl II enzyme by NBS measured in terms of xylanase activity was found to be dependent on both time and inhibitor concentration and followed first order kinetics (Fig. 3). Complete inactivation of Xyl II activity was due to oxidation of one tryptophan residue (Fig. 3, inset). The effect of NBS on xylanase activity showed that after each addition of NBS there was a progressive decrease in absorption at 280 nm with concomittant loss in enzyme activity (data not shown) Fig. 4 shows the residual enzymatic activity plotted against the number of tryptophan groups modified. The extrapolation with the Y-intercept passing through 0% inactivation showed the presence of one tryptophan residue at the catalytic site. The second order rate constant was determined to be 9700 $M^{-1}min^{-1}$. The values of Vmax for the native and modified enzyme was 0.742 μmoles/min/mg and 0.1886 μmmoles/min/mg respectively. The k_m values for the modified enzyme (5mg/ml) increased as compared to the native enzyme (3.5mg/ml) indicating the decreased affinity of the enzyme to the substrate which apparently affected the efficiency of the modified enzyme. Xylan (0.5%) was able to give 100% protection to the enzyme from NBS inactivation (Table 1). The modification of Xyl II was also carried out with HNBB which

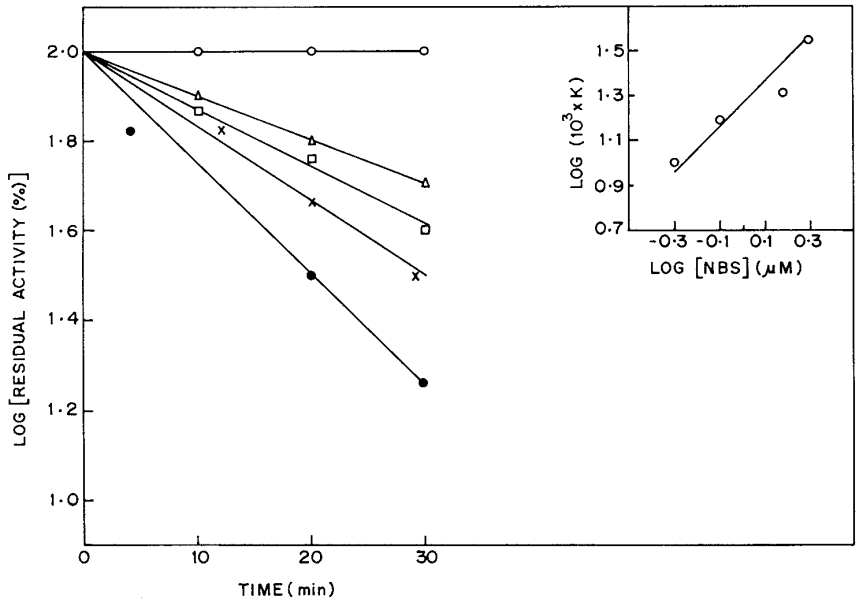

FIG. 3. Kinetics of xylanase from the extremophilic *Bacillus* by NBS:Xylanase was incubated with NBS 0 μM (○), 0.5 μM (△), 1.0 μM (□), 1.5 μM (×), and 2.0 μM (●) at 25°C. Aliquots of the reaction were removed at indicated time intervals. The residual xylanase activity was measured and expressed as percentage of control. Inset: Apparent order of reaction with respect to NBS concentration.

is a more specific reagent for tryptophan. The loss of activity in this case was also due to one tryptophan residue and the second order rate constant was determined to be 33 M^{-1} min^{-1}. The amino acid profile of the chemical modified enzyme was similar to that of the native enzyme.

Assessment of the Microenvironment of Tryptophans

The fluorescence characteristics of tryptophan residues depend strongly on the microenvironment and thus provide a sensitive probe of the conformational state of the protein. Fluorescence quenching of proteins in solutions have been widely used for studying the degree of exposure and electronic environment of aromatic amino acid residues. Acrylamide is an efficient quencher of tryptophan fluorescence and can distinguish between buried and exposed side chains. In contrast, KI and CsCl are highly hydrated, charged molecules and their quenching ability is limited to surface exposed tryptophans and also depends upon the neighbouring charged groups [14,15]. The Stern-Volmer plots of native and Gdn HCl treated Xyl II with various quenchers are shown in (Fig. 5, 6A, 6B). The quenching of Xyl II with increased concentration of acrylamide resulted in a Stern-Volmer plot with an upward curvature, whereas those for CsCl and KI were linear. The static parameter V was calculated to be 1.0M^{-1} for acrylamide. The $K_{(Q)(eff)}$ and f_a values were extrapolated from a replot of the quenching data according to modified Stern-Volmer equation (Fig. 7). The K_{sv} and f_a values are given in Table II. The fluorecence emission of XylII in 6M Gdn HCl shifted to 350nm. The Ksv values for acrylamide and iodide were increased 10 and 2 fold respectively in presence of 6M Gdn HCl indicating exposure of the fluorophores to solvent whereas in case of CsCl there was no change (Table II). The K_{sv} value for acrylamide was much higher than that of KI and CsCl since it can penetrate into the interior and is an efficient quencher. The ratio of the Stern-Volmer constant of tryptophan to that of a protein using the same quencher is the steric factor. In the expression [17]

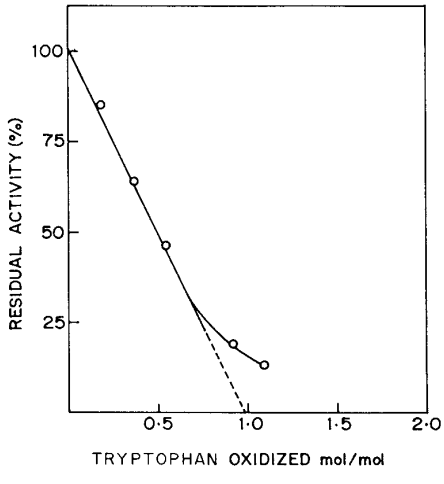

FIG. 4. Plot of percentage residual activity against number of tryptophan groups modified as deduced by spectroscopic studies.

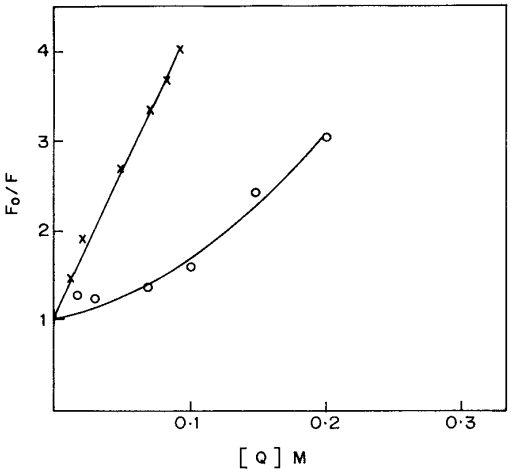

FIG. 5. Stern–Volmer plots of quenching of fluorescence by acrylamide:native (○) and 6 M Gdn HCl-treated enzyme (×).

$$R = (K_{sv}(\text{protein})/K_{sv}(\text{tryptophan}))\ 100\%,$$

the steric factor, R, represents the percent accessibility of the tryptophan residues of Xyl II relative to that of free tryptophan. Since iodide and cesium are oppositely charged, the fluorophore accessibility is appropriately defined as:

$$R = R(I^-) + R(Cs^+)/2.$$

From Fig(7,8) the steric value R for XylII are 10 with iodide ion and 80 with Cs^+ ion. The average steric value is 48, which indicates that 48% of the tryptophans in XylII are exposed to solvent and quencher.

DISCUSSION

The kinetic analysis revealed the participation of one tryptophan residue at the active site of the enzyme. Treatment of Xyl II with NBS results in complete loss of activity and quenching of fluorescence with no change in the emission maximum indicating that inactivation is not due to conformational change in the enzyme. Substrate protection studies by kinetic analysis indicated the involvement of a tryptophan residue at the substrate binding site. This was further confirmed using fluorometric analysis showing 35% protection against loss in fluorescence indicating that not all the tryptophans are involved in the substrate binding site. It has been suggested that interaction between substrate and tryptophan stabilises the transition state during enzymatic hydrolysis of glycosidic bond [18]. The stacking interaction of tryptophan with the face of a xylose ring is similar to a predicted stacking interaction of Trp 457 with a glucose residue at the subsite D in the CelD active site. The stacking interaction is supposede to be a common binding interaction in glycosidases [19]. The tryptophan residues in XylII from

TABLE 1

Protection of Xylanase from Inactivation

Addition	Residual activity (%)
None	100
NBS*	0
Xylan (0.2 mg) + NBS	50
Xylan (0.5 mg) + NBS	80
Xylan (1.0 mg) + NBS	100

* NBS concentration was 5 μm.

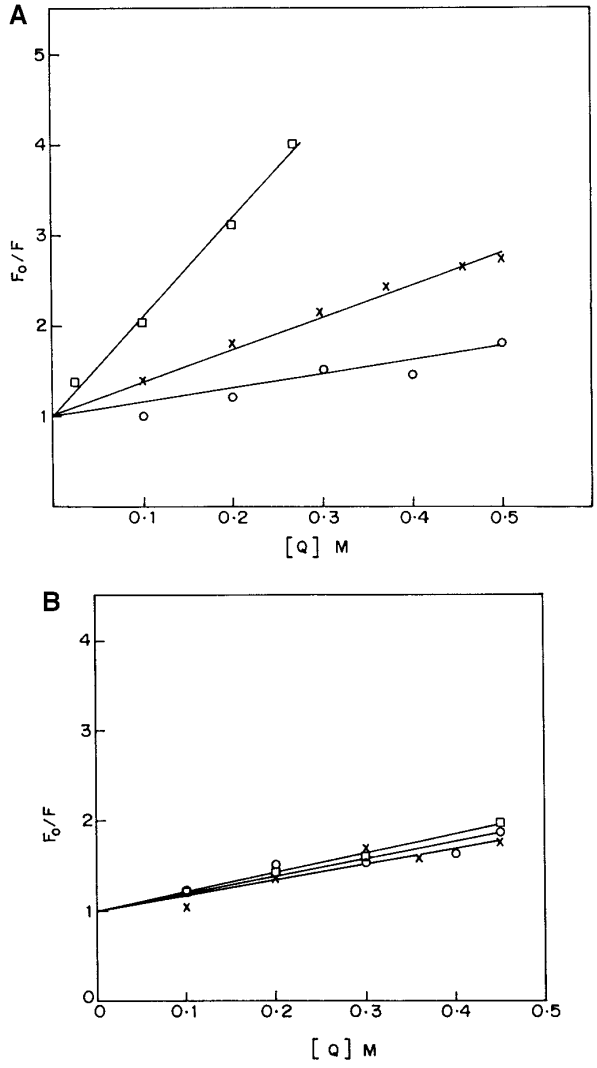

FIG. 6. Microenvironment of xylanase: Stern–Volmer plots. Fluorescence intensities of free tryptophan (□), of xylanase (○), and of enzyme treated with 6 M Gdn HCl (×) are shown. (A) Quenching with I^-; (B) quenching with Cs^+.

the extremophilic Bacillus showed a fluorescence maximum of 339nm, typical of proteins containing tryptophan residues in an hydrophobic environment. In general when the protein unfolds, it exposes buried tryptophan to the aqueous solvent, this results in the shift of fluorescence emission maximum toward longer wavelength. Upon unfolding in 6M GdnHCl the fluorescence emission maxima of XylI shifts to 350nm characteristic of tryptophans fully exposed to water. Further insight into the tertiary structure of XylII may be obtained from fluorescence quenching experiments. KI and CsCl have been suggested as companion probes since these bear opposite charges and their relative quenching efficiencies should be related not only to the accessibility of the protein fluorophore but also to the net charge in the vicinity of the fluorophore. In presence of acrylamide, XylII showed an upward curving Stern-Volmer plot, whereas linear plots with KI and CsCl indicating that the tryptophans may be present in an homogenous environment and that the side chains of all tryptophans have similar collisional constants and the acrylamide quenching of the XylII tryptophan fluorescence proceeds via both a dynamic process. The magnitude of V is related to the probability of finding the quencher molecule close to the fluorophore at the moment of excitation to quench it immediately or statically [16]. When V is less than 2.0^{-1} as is in this case, the steady-state concentration of the quencher in the 'active' volume surrounding the indole ring is very low. This can be explained by a restricted accessibility of the quencher to the side chains of the tryptophans. A linear Stern-Volmer plot with KI and CsCl indicates that no static quenching was observed as is usually is the case. The larger steric value obtained with Cs^+ ion compared to I^- ion as quencher suggests that the tryptophan(s) are located in a relatively negatively charged environment which may be due to the tryptophan(s) being present in the close vicinity of a carboxylic acid residue. Our experiments have indicated the presence of essential carboxylic groups at the active site of XylII with high pka value (6.9) [20] which may be responsible for the activity of the enzyme at alkaline pH. Analysis of the nucleotide sequence of Xyl II (accession number U83602 in the GenBank database) based on the multiple alignment has shown Trp 61 to be highly conserved (manuscript under preparation) and may play a crucial role in the interaction with the substrate. In lysozyme, belonging to the family of glycanases the hydrophobicity of Trp 108 is reported to play an important role in maintaining stability and high pKa value of Glu 35 [21]. NMR assignments of xylanases from *B. circulans* indicate the participation of Trp 71 in a hydrogen bond important for the structure of the active site which probably corresponds to Trp 61 in Xyl II. This interaction conserved in all low molecular weight xylanases of known structure play an important role in establishing the active site conformation of these enzymes [22].

TABLE 2

Quenching Parameters

	Acrylamide			K_{sv}	
	K_{sv}	f_a	K_Q	I^-	Cs^+
Native Xyl I	5.2	1	3.33	1.5	2.0
Xyl II in 6 M Gdn HCl	40.0	1	37.50	2.8	2.0
Free tryptophan	—	—	—	12.5	2.5

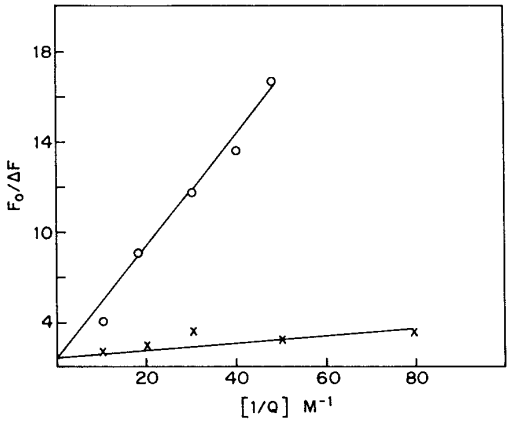

FIG. 7. Modified Stern–Volmer plot of xylanase due to acrylamide quenching: native (○) and in presence of Gdn HCl (×).

ACKNOWLEDGMENT

D.S.N. is a Senior Research Fellow of the Council of Scientific and Industrial Research, India.

REFERENCES

1. Biely, P. (1985) *Trends Biotechnol.* **3,** 286–290.
2. Vernon, C. A. (1967) *Proc. R. Soc. London. B* **167,** 378–388.
3. Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967) *Proc. R. Soc. London B* **167,** 365–377.
4. Wakarchuk, W. W., Campbell, R. L., Sung, W. L., Davoodi, J., and Yaguchi, M. (1994) *Protein Sci.* **3,** 467–475.
5. Henrissat, B., and Bairoch, A. (1993) *Biochem. J.* **3,** 293, 781–788.
6. Dey, D., Hinge, J., Shendye, A., and Rao, M. (1992) *Can. J. Microbiol.* **38,** 436–442.
7. Kulkarni, N., and Rao, M. (1996) *J. Biotechnol.* **51,** 167–173.
8. Shendye, A., and Rao, M. (1993) *FEMS. Microbiol. Lett.* **108,** 297–302.
9. Shendye, A., and Rao, M. (1995) *Biochem. Biophys. Res. Commun.* **195,** 776–784.
10. Miller, G. L. (1959) *Anal. Chem.* **31,** 426–428.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.

12. Levy, H. M., Leber, P. D., and Ryan, E. M. (1963) *J. Biol. Chem.* **238,** 3654–3659.
13. Spande and Witkop, B. (1967) *Methods Enzymol.* **11,** 498–506.
14. Lehrer, S. S. (1971) *Biochemistry* **10,** 3254–3263.
15. Eftink, M. R., and Ghiron, C. A. (1981) *Anal. Biochem.* **114,** 199–227.
16. Goodwin, T. W., and Morton, R. A. (1946) *Biochem. J.* **40,** 628.
17. Pownall, H. J., and Smith, C. L. (1974) *Biochemistry* **13,**
18. Sierks, M. R., Ford, C., Reilly, P. J., and Sevensson, B. (1990) *Prot. Eng.* **3,** 193–198.
19. Juy, M., Amit, A. G., Alazri, P. M., Poljak, R. J., Clayssens, M., Beguin, P., and Aubert, J. P. (1992) *Nature* **357,** 89–91.
20. Chauthaiwale, J., and Rao, M. (1994) *Biochim. Biophys. Acta* **1204,** 164–168.
21. Inoue, M., Yamada, H., Yasukochi, T., Kuroki, R., Miki, T., Horichi, T., and Imoto, T. (1992) *Biochemistry* **31,** 5545–5553.
22. Plesniak, L. A., Wakarchuk, W. W., and McIntosh, L. F. (1996) *Protein Sci.* **5,** 1118–1135.