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Chemical Modification of Specific Active Site Amino Acid Residues of *Enterobacter aerogenes* Glycerol Dehydrogenase

ANJALI PANDEY* and LEELA IYENGAR

Department of Chemistry, Indian Institute of Technology, Kanpur 208016, India

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Enterobacter aerogenes glycerol dehydrogenase (GlDH EC 1.1.1.6), a tetrameric NAD^+ specific enzyme catalysing the interconversion of glycerol and dihydroxyacetone, was inactivated on reaction with pyridoxal 5′-phosphate (PLP) and _o_-phthalaldehyde (OPA). Fluorescence spectra of PLP-modified, sodium borohydride-reduced GlDH indicated the specific modification of ε-amino groups of lysine residues. The extent of inhibition was concentration and time dependent. NAD^+ and NADH provided complete protection against enzyme inactivation by PLP, indicating the reactive lysine is at or near the coenzyme binding site. Modification of GlDH by the bifunctional reagent OPA, which reacts specifically with proximal ε-NH_2 group of lysines and -SH group of cysteines to form thioisoindole derivatives, inactivated the enzyme. Molecular weight determinations of the modified enzyme indicated the formation of intramolecular thioisoindole formation. Glycerol partially protected the enzyme against OPA inactivation, whereas NAD^+ was ineffective. These results show that the lysine involved in the OPA reaction is different from the PLP-reactive lysine, which is at or near the coenzyme binding site. DTNB titration showed the presence of only a single cysteine residue per monomer of GlDH. This could be participating with a proximal lysine residue to form a thioisoindole derivative observed as a result of OPA modification.

Keywords: Glycerol dehydrogenase; Pyridoxal phosphate; *o*-Phthalaldehyde; Thioisoindole; Enzyme inactivation

INTRODUCTION

Glycerol dehydrogenase (EC.1.1.1.6) is a NAD^+-dependent enzyme which catalyses the interconversion of glycerol and dihydroxy acetone. Although the metabolic role of glycerol dehydrogenase is not well defined, recent interest in this enzyme has been raised by the possibility of incorporating this enzyme in diagnostic assays for serum triglyceride after hydrolysis with a lipase. Glycerol dehydrogenase (GlDH) has been isolated from both microbial and animal sources.[1–5] However, there have been only few reports of its characterization including the nature of amino acids present at the active site. Spencer *et al*..[3,6] purified GlDH from a thermophilic bacteria, *Bacillus stearothermophilus*, which required bound zinc for activity. Further, they identified a structurally important cysteine residue using thiol-specific chemical modifiers. The presence of a lysine at the nucleotide binding site was reported for the same enzyme using pyridoxal phosphate.[7]

In the present investigation, a chemical modification approach using two modifiers, pyridoxal phosphate (PLP) and *o*-phthalaldehyde (OPA), has been used to identify the active site amino acids of *Enterobacter aerogenes* glycerol dehydrogenase.

EXPERIMENTAL PROCEDURES

Materials

Purified *E. aerogenes* glycerol dehydrogenase, NAD^+, NADH, PLP and *o*-phthalaldehyde were purchased from Sigma Chemical Company USA. Glycerol, buffer components and other chemicals used were of highest purity grade.

Enzyme Assay

Glycerol dehydrogenase activity was determined spectrophotometrically by measuring the increase in absorbance at 340 nm using a Shimadzu UV-visible

*Corresponding author. Fax: +91-512-597436. E-mail: anjali@iitk.ac.in.

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spectrophotometer. The reaction mixture contained 35 mM ammonium sulphate, 35 mM NAD^+, 100 mM glycerol, 100 mM carbonate buffer (pH 10) and 0.1 μM of enzyme in a total volume of 1 ml. One unit of enzyme is defined as the amount of enzyme required to oxidize 1 μmole of glycerol to dihydroxy acetone at 25°C, and the specific activity of the purified enzyme used in the present study was 25 units mg^{-1} protein. Protein determination was carried out by the method of Lowry *et al.*[8]

Modification With PLP

Enzyme (0.1 μM) was incubated with various concentrations of PLP (2–15 mM) in 0.05 M potassium phosphate buffer, pH 7.0, which was protected from light. Aliquots were removed after incubating at room temperature for 20 min and residual activity was determined. Appropriate controls without added inhibitor were always included.

Reduction of the Schiff's Base With Sodium Borohydride

The enzyme (1.0 μM) was incubated with 5 mM PLP for 20 min and then the Schiff's base was reduced by adding 20-fold molar excess of $NaBH_4$.[9] The reaction mixture was kept in ice for 30 min and then dialysed extensively against 0.05 M phosphate buffer (pH 7.0). Aliquot was taken for the spectral analysis.

Modification With OPA

An *o*-phthalaldehyde solution was prepared in 1% methanol for modification reactions. Enzyme (0.1 μM) was incubated with different concentrations of OPA (200–500 μM) at room temperature. Aliquots were withdrawn at various intervals and assayed for residual activity. Control experiments were carried out under similar conditions in the absence of OPA.

Protection Experiments

For protection experiments against PLP inactivation of the enzyme, Glycerol (200 mM), NAD^+ (2 mM) and NADH (0.05 mM) were used as protecting agents against enzyme inactivation. Protecting ligands were preincubated with the enzyme for 5 min prior to the addition of PLP (10 mM). Aliquots were taken at various times for the determination of residual enzyme activity. Controls containing the protecting ligands, without PLP, were kept for each reaction. For protection studies with OPA glycerol (100 and 200 mM) or NAD^+(1 and 2 mM) were preincubated with the enzyme for 5 min prior to the addition of OPA (400 μM). Aliquots were taken after 20 min for the determination of residual enzyme activity.

Fluorescence Spectroscopy

Fluorescence emission spectra of PLP-modified and $NaBH_4$-reduced enzyme as well as OPA-modified enzymes were recorded on a Perkin–Elmer fluorometer (Model LS50B) using excitation wavelengths of 325 and 337 nm, respectively.

The stoichiometry of NADH binding to the native, and PLP-modified, and $NaBH_4$-reduced enzyme was determined in the same instrument using an excitation wavelength of 340 nm and monitoring the emitted light at 430 nm. NADH (0.1 mM) was titrated into either 1 ml buffer, 1 ml of 2.26 μM native enzyme or 2.26 μM modified enzyme. A correction factor was applied to the measured fluorescence intensity with the modified enzyme as PLP-modified and $NaBH_4$ reduced enzyme contributed a small amount of fluorescence at 430 nm even in the absence of NADH. The difference between the native and modified enzymes after subtracting the fluorescence intensity observed with the buffer control, was used to assess the effect of modification on NADH binding.[10]

Enzyme Denaturation

Denaturation of GlDH was carried out using 6 M urea for 30 min in 0.05 M phosphate buffer (pH 7.5). Heat denaturation of GlDH was carried out by keeping the enzyme in boiling water bath for 5 min.

Gel Electrophoresis

Presence of SDS results in disruption of tetrameric *E. aerogenes* GlDH to monomeric subunits. SDS polyacrylamide gel electrophoresis of native as well as *o*-phthalaldehyde-modified enzyme was carried out by Laemmli's method.[11]

Determination of Thiol Group In *E. Aerogenes* Glycerol Dehydrogenase

Cysteine residues of GlDH were determined by the procedure described by Habeeb.[12] Protein was denatured for 5 min at room temperature by addition of 2% SDS in buffer solution (pH 8.0). DTNB (0.67 mM) was added to this solution, the mixture kept for 20 min and then the absorbance at 412 nm recorded. The molar extinction coefficient for calculation was taken to be 13,600/cm^{-1}.

RESULTS AND DISCUSSION

Enzyme Modification With PLP

Incubation of glycerol dehydrogenase with PLP led to the inactivation of the enzyme. The kinetics of inactivation at different concentrations (2–15 mM) of

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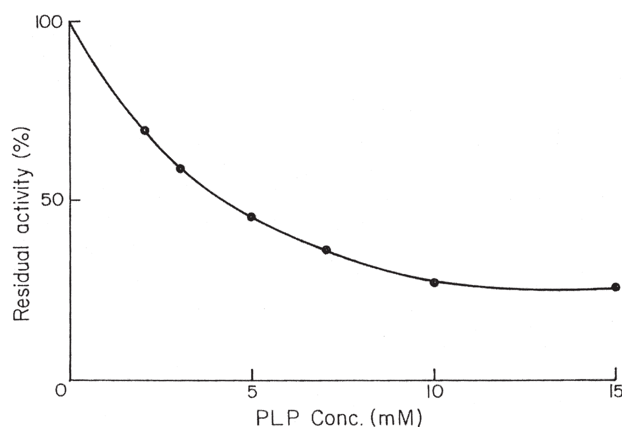

FIGURE 1 The concentration dependence of the PLP inactivation of glycerol dehydrogenase. The enzyme (0.10 μM) was incubated with different concentration (2–15 mM) of PLP for 20 min. Aliquots (10 μl) were withdrawn for assay, and residual activity was determined as described in the "Experimental procedures" section.

PLP demonstrated that the inactivation of the enzyme reached a constant non-zero value after 15 min. However, residual enzyme activity decreased with increasing concentration up to 10 mM PLP, beyond which there was only a marginal enhancement in inactivation (Fig. 1).

Residual enzymatic activity after PLP treatment, has been reported for many enzymes.[13,14] Chen and Engel[15] have proposed a model to explain this lack of complete inactivation by PLP as shown below:

$$E + PLP \leftrightarrow E.PLP(\text{Noncovalent Complex}) \leftrightarrow E{-}PLP(\text{Covalent Complex}) \quad (1)$$

Enzyme lysine residues react with PLP, leading to the formation of a covalent Schiff's base, via a noncovalent intermediate. The residual activity observed is due to the reversibility of both of these reactions.

The fluorescence spectrum of PLP-modified sodium borohydride-reduced enzyme recorded at an excitation wavelength of 325 nm showed an emission maximum at 395 nm. This is characteristic of the modification of ε-amino groups of lysine of the enzyme[9] (Fig. 2).

Protection experiments were carried out in order to determine the probable site of modification. Glycerol, NAD^+ and NADH were used as protecting lig1ands. Results showed that the inactivation of GlDH by added PLP was completely prevented in the presence of either NAD^+ or NADH (Fig. 3). The extent of NADH binding, determined fluorimetrically, to the PLP-treated (2 mM) and $NaBH_4$ reduced enzyme was only 60% when compared to the native enzyme. The residual activity of the same enzyme was 65% of the control. This data indicates that the loss in NADH binding is stoichiometrically correlated with the loss in enzyme activity. There was no significant protection of GlDH against PLP inactivation in the presence of glycerol. These results demonstrate that the PLP-reactive lysine residue responsible for enzyme inactivation is at, or very near, the coenzyme binding site. Paine *et al.*[7] have reported similar observations with the *B. stearothermophilus* GlDH.

Enzyme Inactivation With *o*-phthalaldehyde

The kinetics of inactivation of GlDH by different concentrations of OPA is shown in Fig. 4. The logarithmic plot of inactivation, versus incubation time with *o*-phthalaldehyde, yields a linear relationship in a concentration range of 200–500 μM, indicating first order inactivation kinetics. The rate

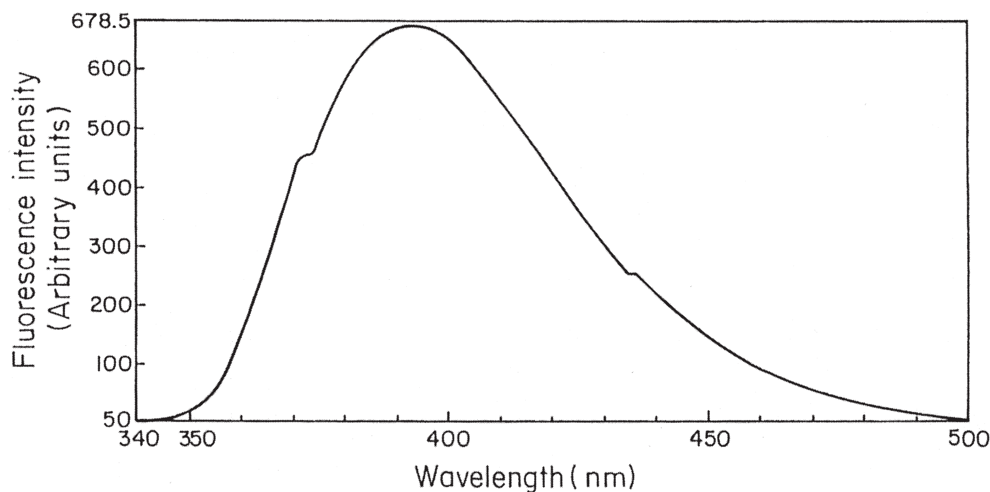

FIGURE 2 Fluorescence emission spectrum of pyridoxal phosphate-modified and sodium borohydride-reduced glycerol dehydrogenase. The enzyme (1.0 μM) was incubated with 5 mM PLP for 20 min in 0.05 M potassium phosphate buffer (pH 7.5) at 25°C and then reduced by sodium borohydride. The emission spectrum of the reduced enzyme–PLP complex with excitation at 325 nm was recorded as described in the "Experimental procedures" section.

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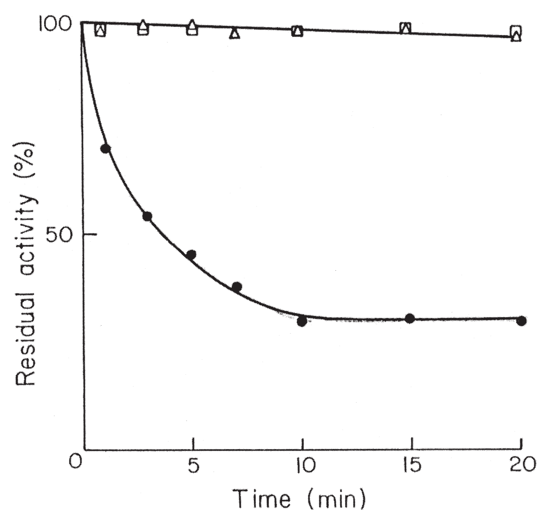


FIGURE 3 The inactivation of glycerol dehydrogenase by PLP in the presence or absence of coenzyme. The enzyme (0.10 μM) was incubated with 10 mM PLP in the absence (•) or presence of 0.05 mM NADH (□) or 2 mM of NAD^+ (△). Aliquots (10 μl) were withdrawn at various time intervals for activity determination as described in the "Experimental procedures" section.

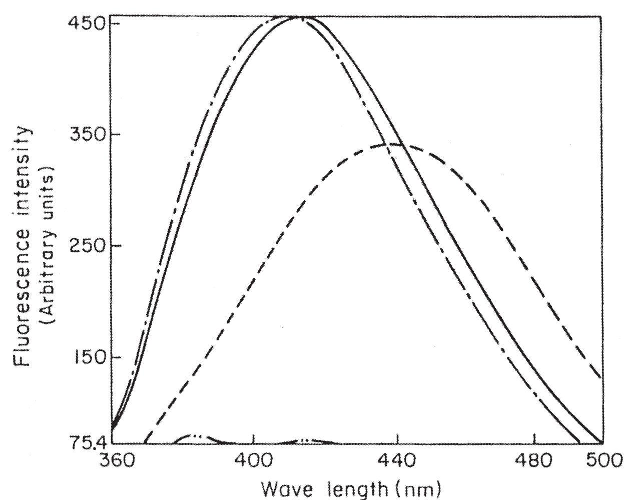


FIGURE 5 Fluorescence emission spectrum of *o*-phthalaldehyde-modified glycerol dehydrogenase. The enzyme (0.10 μM) was incubated with 400 μM OPA for 20 min in 0.05 M phosphate buffer (pH 7.5) at 25°C. The emission spectrum with excitation at 337 nm (—) was recorded as described in the "Experimental procedures" section. Denaturation of the enzyme was carried out using 6 M urea for 30 min at 25°C in 0.05 M potassium phosphate buffer (—••—). Urea denatured enzyme treated with 400 μM *o*-phthalaldehyde for 30 min (------). The enzyme was denatured by heat treatment for 5 min and subsequently with *o*-phthalaldehyde (—•—).

of inactivation was dependent on the concentration of *o*-phthalaldehyde, and 88% inactivation was observed within 8 min with 500 μM *o*-phthalaldehyde. The fluorescence emission spectrum of GlDH after modification with *o*-phthalaldehyde was recorded between 360–500 nm using an excitation wavelength of 337 nm (Fig. 5); the *o*-phthalaldehyde-modified enzyme showed an emission maximum at 415 nm. This is characteristic of thioisoindole derivatives formed from the reaction of OPA with the -SH group of cysteine residue and ε-NH_2 group of lysine residues in the enzyme.[16,17] The following equation is given for thioisoindole derivative formation from enzyme cysteine -SH group and ε-amino group of lysine with OPA.

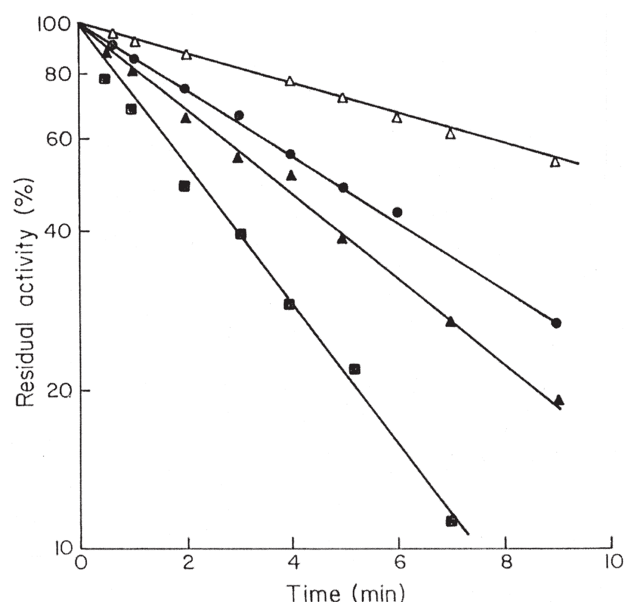
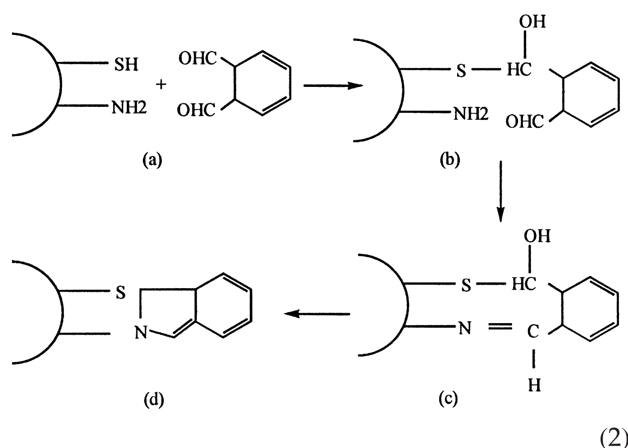


FIGURE 4 Pseudo First order kinetic plot for the inactivation of glycerol dehydrogenase by *o*-phthalaldehyde. The enzyme (0.10 μM) was incubated with different concentrations of OPA (△) 200 μM; (•) 300 μM; (▲) 400 μM; (■) 500 μM. Aliquots (10 μl) were withdrawn at different time intervals and the residual activity was determined as described in the "Experimental procedures" section.



(2)

Molar transition energy for *E. aerogenes* GlDH enzyme OPA reaction was calculated to be 151.5 kJ mol^{-1}, using the following relationship.[16] $E_T = 2.985\lambda_{em} - 1087.28$. The E_T value has been used by many investigators[18,19] to probe the polarity of the microenvironment of participating cysteine and lysine residues by comparing it with the values of a synthetic thioisoindole OPA adduct in different solvents. E_T for the adducts has been reported to be 127 kJ mol^{-1} in hexane, 155 kJ mol^{-1} in dioxane and

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TABLE I Effect of substrates on the inhibition of glycerol dehydrogenase by *o*-phthalaldehyde

Protecting compound	Concentration (mM)	Residual activity (%)
None	–	20
NAD^+	1	22
	2	24
Glycerol	100	56
	200	60

NAD^+ or glycerol was preincubated with enzyme (0.1 μM) prepared in 0.05 M potassium phosphate buffer (pH 7.5) before the addition of *o*-phthalaldehyde. 10 μl aliquots were withdrawn after 20 min for assay to determine the residual activity.

267 kJ mol^{-1} in water. The calculated E_T value in the present study for GlDH-OPA was 151.5 kJ mol^{-1}, which is closer to the value in dioxane. This indicates that the microenvironment of the participating cysteine and lysine residues is hydrophobic.

Denaturation of GlDH by either urea or heat treatment prior to *o*-phthalaldehyde modification did not significantly affect the fluorescence intensity. However, the emission maxima shifted from 410 to 440 nm after denaturation with urea (Fig. 5). The calculated E_T value for urea denaturated enzyme was 226 kJ mol^{-1} which shows that the microenvironment was less hydrophobic. Thus, the spatial proximity of the -SH group of cysteine and ε-amino group of lysine is maintained even after denaturation. It may be reasonable to assume that these groups are proximal in the primary structure itself.

Both native and OPA modified GlDH gave a single band with identical mobilities when examined by sodium dodecyl sulfate–polyacrylamide electrophoresis. *E. aerogenes* GlDH has been reported to be a tetrameric enzyme with four identical subunits of 42 kDa[3]. As our results did not show any higher molecular weight after OPA modification, it can be concluded that the reaction involves the formation of crosslinks between cysteine and lysine residues within the same polypeptide chain.

Protection against OPA inactivation, in the presence of either glycerol or NAD^+, indicated that only glycerol partially protected the enzyme against inactivation (Table I). Thus, the participation of the PLP-reactive lysine residue located at or near the coenzyme binding site in isoindole derivative formation is unlikely. DTNB titration of GlDH showed that there is only one cysteine residue per subunit of *E. aerogenes* GlDH, which was absent after modification with OPA.

Spencer *et al.*[6] have reported that there is only one cysteine residue per subunit in *B. stearothermophilus* GlDH, which is reactive to modification by thiol specific reagents, such as DTNB and iodoacetamide, only after zinc-depletion and not in the native enzyme. It has been demonstrated that this cysteine residue plays an important role in subunit–subunit interaction of glycerol dehydrogenase, which is probably required for the enzyme activity. Amino acid sequence analysis of *B. stearothermophilus* GlDH has shown that a lysine residue precedes the sole cysteine.[6]

The present study has shown that there is only one cysteine per monomer in *E. aerogenes* GlDH. This cysteine reacts with OPA leading to inactivation. Assuming that the cysteine and the preceding lysine (*B. stearothermophilus*) are conserved in glycerol dehydrogenase from *E. aerogenes*, it is tempting to suggest that these two adjacent residues may be participating in thioisoindole formation leading to *E. aerogenes* GlDH inactivation.

References

[1] Burton, R.M. and Kaplan, N.O. (1953), *J. Am. Chem. Soc.* **75**, 1005–1006.
[2] Jacobs, N.J. and Vandemark, P.J. (1960), *J. Bacteriol.* **79**, 532–538.
[3] Spencer, P., Brown, K.J., Scawen, M.D., Atkinson, T. and Gore, M.G. (1989), *Biochim. Biophys. Acta* **994**, 270–279.
[4] Korman, A.W., Hurst, R.O. and Flynn, T.G. (1972), *Biochim. Biophys. Acta* **258**, 40–45.
[5] Moore, B.W. (1959), *J. Am. Chem. Soc.* **81**, 5837–5838.
[6] Spencer, P., Scawen, M.D., Atkinson, T. and Gore, M.G. (1991), *Biochim. Biophys. Acta* **1073**, 386–393.
[7] Paine, L.J., Perry, N., Popplwell, A.G., Gore, M.G. and Atkinson, T. (1993), *Biochim. Biophys. Acta* **1202**, 235–243.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951), *J. Biol. Chem.* **193**, 265–275.
[9] Fischer, E.H., Forrey, A.W., Hedrick, R.C., Kent, A.B. and Krebs, E.G. (1963) In: Snell, E.E., Fasella, P.M., Braunstein, A. and Rossi-Fanelli, A., eds, *Chemical and Biological Aspects of Pyridoxal Catalysis* (Pergamon Press, New York), pp 543–562.
[10] Stinson, R.A. and Holbrook, J.J. (1973), *Biochem. J.* **131**, 719–728.
[11] Laemmli, U.K. (1970), *Nature (Lond.)* **227**, 680–685.
[12] Habeeb, A.F.S.A. (1970), *Methods Enzymol.* **25**, 454–464.
[13] Lilly, K.S. and Engel, P.C. (1992), *Eur. J. Biochem.* **207**, 533–540.
[14] Poulose, A.J., Rogers, L. and Kolattukudy, P.E. (1980), *Arch. Biochem. Biophys.* **201**, 313–321.
[15] Chen, S.S. and Engel, P.C. (1975), *Biochem. J.* **149**, 619–626.
[16] Palczewski, K., Hargrave, P.A. and Kochman, M. (1983), *Eur. J. Biochem.* **139**, 429–435.
[17] Sheikh, S., Mukund, K. and Katiyar, S.S. (1993), *Biochim. Biophys. Acta* **1203**, 276–281.
[18] Chen, C.-Y., Emig, F.A., Scharmm, V.L. and Ash, D.E. (1991), *J. Biol. Chem.* **226**, 16645–16652.
[19] Puri, R.N., Bhatnager, D.B. and Roskoski, Jr, R. (1988), *Biochim. Biophys. Acta* **957**, 34–46.

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