Chemical modification of glucose oxidase: possible formation of molten globule-like intermediate structure

Saman Hosseinkhani[a,b], Bijan Ranjbar[b], Hossein Naderi-Manesh[b], Mohsen Nemat-Gorgani[a,*]

[a] *Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145-1384, Tehran, Iran*
[b] *Department of Biochemistry, Faculty of Basic Sciences, Tarbiat Modarres University, P.O. Box 14115-175, Tehran, Iran*

Received 13 January 2004; accepted 16 January 2004

First published online 13 February 2004

Edited by Thomas L. James

Abstract Chemical modification of lysine residues in glucose oxidase was carried out using citraconic anhydride. Modification brought about changes in the kinetic properties of the enzyme as evident by substantial lowering of V_{max} and K_m. Enhancement of tryptophan fluorescence was observed with a dramatic change in its pH dependence due to modification. Near- and far-UV circular dichroism spectra of the native and modified forms suggested formation of molten globule-like structures, further supported by 8-anilino-1-naphthalenesulfonic acid fluorescence which indicated higher exposure of hydrophobic residues as a result of chemical modification.

Key words: Chemical modification; Glucose oxidase; Molten globule; 8-Anilino-1-naphthalenesulfonic acid; Intrinsic fluorescence; Circular dichroism

1. Introduction

Glucose oxidase (β-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to δ-gluconate, coupled with reduction of O_2 to H_2O_2 [1,2]. The enzyme is found in a number of fungal sources and is composed of two identical subunits with 2 mol of flavin adenine dinucleotide (FAD) located inside deep pockets in the protein structure [3]. FAD stabilizes the three-dimensional structure of the enzyme, is bound very tightly and cannot be removed by dialysis at neutral pH [4]. Glucose oxidase from *Aspergillus niger*, used in the present study, is a dimer of MW 160 kDa with a pH optimum of 5.5 and a p*I* of 4.2 [5].

It is now generally accepted that denatured proteins can exist in a wide range of structures in terms of compactness and residual secondary structure. Moreover, some of these forms may contain essentially as much secondary structure as the native state. The term 'molten globule' was first used in the literature by Ohgushi and Wada to describe the acid-denatured state of cytochrome *c* [6]. The denatured protein resembled the compact state with native-like secondary structure but little or no native-like tertiary structure, and a significant exposure of hydrophobic surface [7]. Such compact denatured states have been observed for a large number of proteins, and it is assumed that most, if not all, proteins can form such species if the appropriate experimental conditions can be provided. Accordingly, a number of proteins have been found to be transformed into the molten globule state either at low pH [8] or at intermediate concentrations of guanidine hydrochloride [9].

Chemical modification of proteins has been used as a tool for studying localization of individual amino acids, their participation in the maintenance of the native conformation [10], their stabilization [11] and conversion to molten globule structures [12]. In the present report, modification of ε-amino groups of lysine residues in glucose oxidase has been performed using citraconic anhydride. Changes in the kinetic properties, the circular dichroism (CD) spectra, intrinsic and extrinsic fluorescence suggest formation of molten globule-like structures in the protein brought about by chemical modification. The procedure has already been found useful in connection with increasing exposure of hydrophobic residues in the enzyme for improvement of its interaction with a hydrophobic support [13].

2. Materials and methods

2.1. Materials

Glucose oxidase, citraconic anhydride and all other biochemicals were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical reagent grade. Reproducibility of the data presented in this article was confirmed by repeating the experiments at least twice.

2.2. Protein concentration

Concentrations of native and modified forms of glucose oxidase were determined by the Lowry method [14].

2.3. Fluorescence measurements

Tryptophyl fluorescence was measured on a Hitachi MPF-4 apparatus. The excitation wavelength was set at 295 nm and the emission spectra were obtained. To study the effect of pH on protein fluorescence, a buffer mixture containing glycine (pK_a 2.34, 9.6), succinate (pK_a 4.21), MES (pK_a 6.1) each at 10 mM concentration adjusted to the required pH was used.

Extrinsic fluorescence studies were carried out as outlined earlier [15–17], using 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescence probe. Measurements were taken on the same spectrofluorometer as used for intrinsic fluorescence studies. All experiments were carried out at 25°C with ANS and protein concentrations of 50 μM and 50 μg/ml in 0.1 M phosphate buffer. An excitation wavelength of 350 nm was used.

2.4. Enzyme assay

Enzymatic activity of native and modified glucose oxidase was determined by a colorimetric method using a coupled assay [18,19]. 10 μl glucose solution (18% w/v) and 0.5 U of peroxidase were added to 1.0

*Corresponding author. Fax: (98)-21-6404680.
E-mail address: gorganim@ibb.ut.ac.ir (M. Nemat-Gorgani).

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; FAD, flavin adenine dinucleotide

0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00134-6

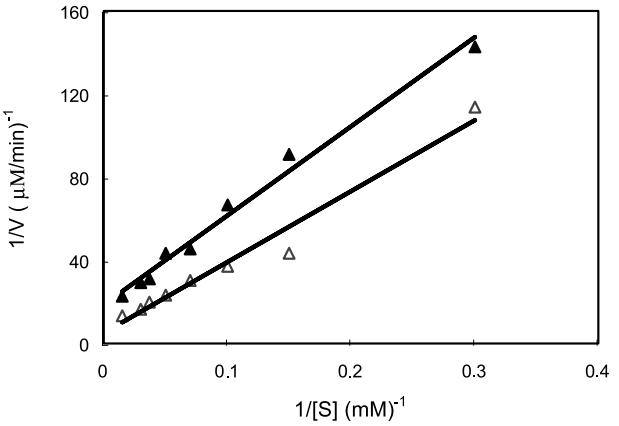


Fig. 1. Double reciprocal plots for native (△) and modified (▲) glucose oxidase at several concentrations of glucose. Further details are described in the text.

ml of 0.1 M phosphate buffer, containing 1% *O*-dianisidine, pH 6.0. The reaction was started by addition of 10 μl of glucose oxidase (2.5 U) and the rate of increase in absorbance at 460 nm was determined.

2.5. Modification of lysine residues

Modification was carried out using citraconic anhydride as a specific blocking agent for lysine residues and following the procedure described by Dixon and Perham [20]. The protein was used at 4 mg/ml concentration in 10 ml of 100 mM borate (pH 8.0) and the process was followed at room temperature by step-wise addition of 3 μl aliquots of the modifier while maintaining the pH of the stirred solution at 8.0 by the addition of 2 M NaOH. The reaction was complete upon addition of 15 μl of citraconic anhydride in a total time of 30 min, at which point the pH of the solution remained stable. The reaction mixture was then dialyzed extensively against 100 mM phosphate, pH 6.0. Isoelectric focusing runs were carried out according to O'Farrell [21].

To determine the number of modified lysine residues, the number of free amino groups was measured following the method described by Fields [22]. The sample was first dialyzed in 0.1 M borate buffer, pH 9.5 and the final protein concentration was adjusted to 0.1 mg/ml. From a total of 30 lysine residues about 15 residues were found to be modified following the procedure described above.

2.6. Electrophoretic procedure

Sodium dodecyl sulfate gel electrophoresis (SDS–PAGE) was performed using 13.5% acrylamide gel without β-mercaptoethanol according to Laemmli [23]. Gels were stained with Coomassie brilliant blue R250 and, when desired, scanned using a Hellena densitometer.

2.7. CD measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with protein concentrations about 0.2 and 1.5 mg/ml for far- and near-UV regions respectively. Results are expressed as molar ellipticity, $[\theta]$ (deg cm^2 dmol^{-1}), based on a mean amino acid residue weight (MRW) of 110 for glucose oxidase. The molar ellipticity was determined as $[\theta]_\lambda = (\theta \times 100\mathrm{MRW})/(cl)$, where c is the protein concentration in mg/ml, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at a wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2 dmol^{-1}, and with JASCO standard non-hydroscopic ammonium (+)-10-camphorsulphonate, assuming $[\theta]_{290.5} = 7910$ deg cm^2 dmol^{-1} [24]. The data were smoothed using the fast Fourier transform noise reduction routine which allows enhancement of most noisy spectra without distorting their peak shapes.

3. Results and discussion

Modification of lysine residues was carried out using citraconic anhydride and following the procedure described in Section 2. Initial activity measurements showed that the enzymatic potential is substantially diminished upon modification. Analysis of kinetic data presented in Fig. 1 indicated that the loss of activity coincides with an appreciable decrease in K_m in addition to V_{max}: the K_m from 66.6 mM to 22.7 mM and the V_{max} from 0.2 to 0.05 μmol/min. These alterations in kinetic parameters suggested possible structural changes in the protein molecule upon modification. SDS–PAGE, run under non-reducing conditions, indicated that modification does not alter the association state of the protein. The far-UV and near-UV CD spectra of the native and modified forms were also taken which reflected substantial changes in the tertiary structure of the protein without significant changes in its secondary structure (Fig. 2). A clear improvement in conformational stability of the protein was observed upon modification. This was suggested by lowering of ellipticity as indicated by near-UV CD spectra taken in the presence of high concentrations of urea (Fig. 3) and an increase in T_m of 3.5°C. Another approach taken in this investigation involved intrinsic fluorescence studies. As indicated in Fig. 4 enhancement of fluorescence coincided with a red shift upon modification of the enzyme. Furthermore, the nature of the dependence of fluorescence on pH was dramatically altered (Fig. 5). For the native enzyme, the spectra taken at various pH values indicated red shifts of the emission spectra in acid and alkaline pH ranges (results not shown), with minimum fluorescence at pH 6.0. On the other

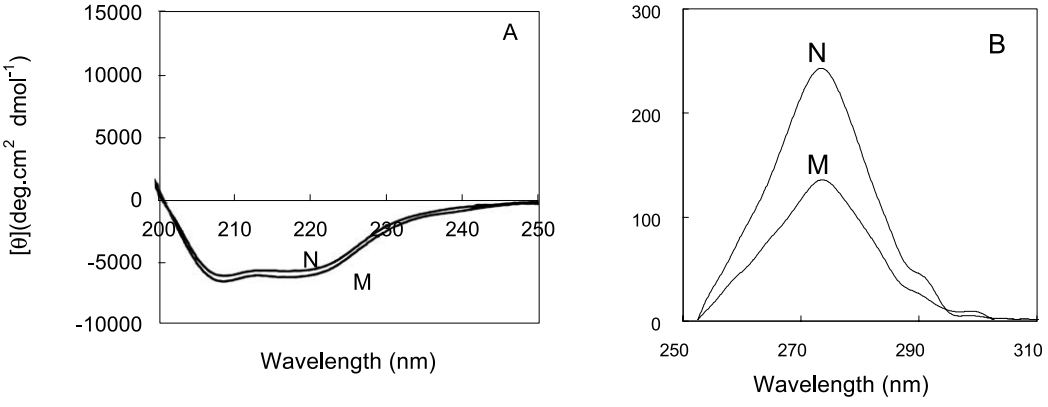


Fig. 2. Far-UV CD spectra (A) and near-UV CD spectra (B) for native (N) and modified (M) glucose oxidase. For further details please see Section 2.

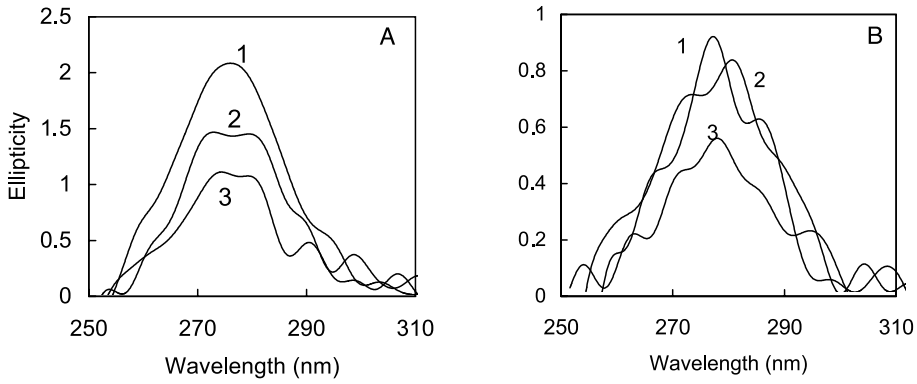


Fig. 3. Near-UV CD spectra of native (A) and modified (B) glucose oxidase in the presence of different concentrations of urea. (1) 0 M urea, (2) 2 M urea, (3) 4 M urea.

hand, the modified form showed a maximum amount of fluorescence at this pH.

Modification of ε-amino groups of lysine residues (Scheme 1) alters a positive charge (p$K \approx 10$) to a negative charge (carboxylic group, with a much lower pK). Thus at pH 6.0, at which most of the experiments were carried out, there is clearly the possibility for a reverse of charge at the modified residues as also confirmed by a decrease in the pI of the native protein upon its treatment with citraconic anhydride. It may therefore be suggested that the dramatic change in pH dependence of intrinsic fluorescence (Fig. 5) occurs as a result of protein modification due to changes in the net charge, by introduction of additional negative charges in the protein molecule (Scheme 1).

Accordingly, it is assumed that protonation or ionization of the titratable groups of certain amino acids (such as tyrosine, lysine and arginine), considered important in this connection, is significantly altered [25,26]. This would result in a different pattern of conformational changes in the protein structures (native and modified) with respect to changes in pH, followed by alteration of the microenvironment of excitable tryptophan residue(s), as supported by an obvious change in the emission maximum (Fig. 4). Alternatively, the results presented may be explained in terms of the quenching effect of charged carboxyl and/or amino groups. Thus for the native enzyme, it is proposed that pH values relatively more acidic or more alkaline than pH 6 result in these quenchers being displaced farther away from the tryptophan residue(s) and thereby in fluorescence enhancement. For the modified enzyme, the situation is reversed due to changes brought about by its modification discussed above (Scheme 1). A similar pattern in the dependence of the fluorescence of the native protein on pH changes, observed here, has been reported for anticoagulation factor I from *Agkistrodon acutus* venom [27].

The type of changes observed in the CD spectra upon modification of glucose oxidase suggests possible formation of molten-like structures (Fig. 2). The transition to the molten globule state has been shown to be accompanied by loss of tertiary interactions whereas most of the secondary structure is preserved. This is manifested by the disappearance of the CD bands in the near-UV region and the virtually unchanged CD spectrum in the far-UV region [28,29]. It has been established that the semi-flexible structure of the molten globular state permits exposure of hydrophobic groups in the protein structure. Accordingly, to test a higher exposure of the hydrophobic sites upon modification of the enzyme, ANS was used as a hydrophobic reporter group. This probe has been found to provide a particularly useful test for the molten globule state [16]. It is bound to the equilibrium molten globule

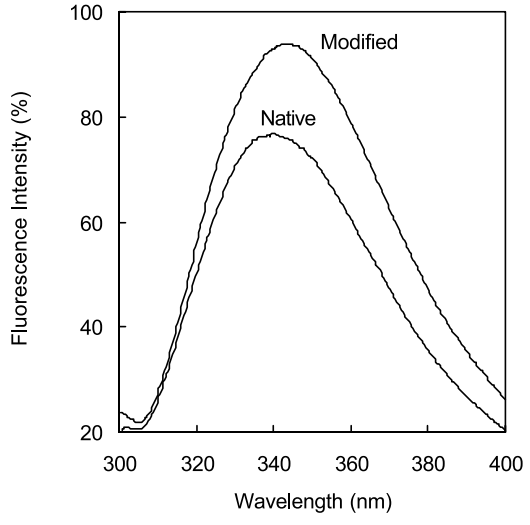


Fig. 4. Effect of lysine modification on intrinsic fluorescence. The tryptophan fluorescence spectra for native and modified forms were measured upon excitation at 295 nm at 25°C. Further details are provided in Section 2.

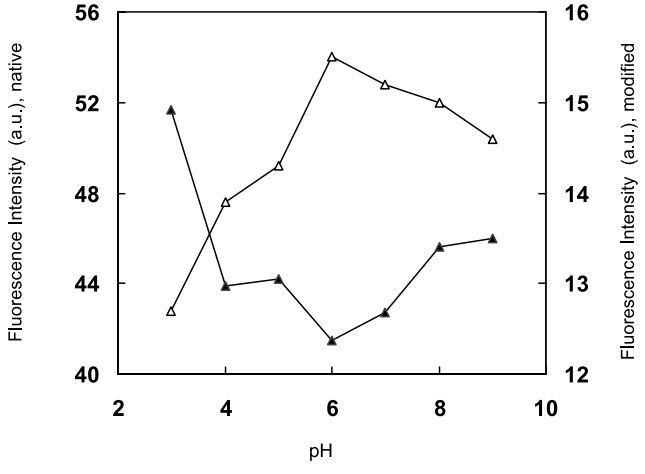
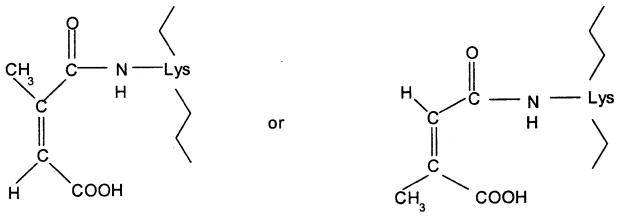


Fig. 5. pH dependence of intrinsic fluorescence for native (△) and modified (▲) glucose oxidase. For additional details, see legend to Fig. 3 and Section 2.



Scheme 1.

much more strongly than the native and the unfolded states with a dramatic increase in its fluorescence intensity [15–17].

In the present study we observed, as expected, a clear enhancement of ANS fluorescence upon modification of glucose oxidase (Fig. 6). However, the typically high enhancement reported for other proteins [30,31] was not observed here. This is probably due to the fact that ANS itself carries a negative charge at the pH of the experiment and, upon modification of the enzyme (p*I* 4.2), additional negative charges are introduced (Scheme 1 and p*I* determination). It is therefore suggested that had it not been for such repulsive electrostatic interactions, the typically higher enhancement reported for other proteins would have been obtained. Similar suggestions have been made in the literature [32]. Some quenching of fluorescence by FAD may also occur.

In conclusion, the results presented in this study suggest formation of molten globule-like intermediate structures in glucose oxidase upon its chemical modification.

Acknowledgements: The authors thank Mrs. Atieh Ghasemi for her technical assistance and Mrs. Leila Hassani for taking CD spectra.

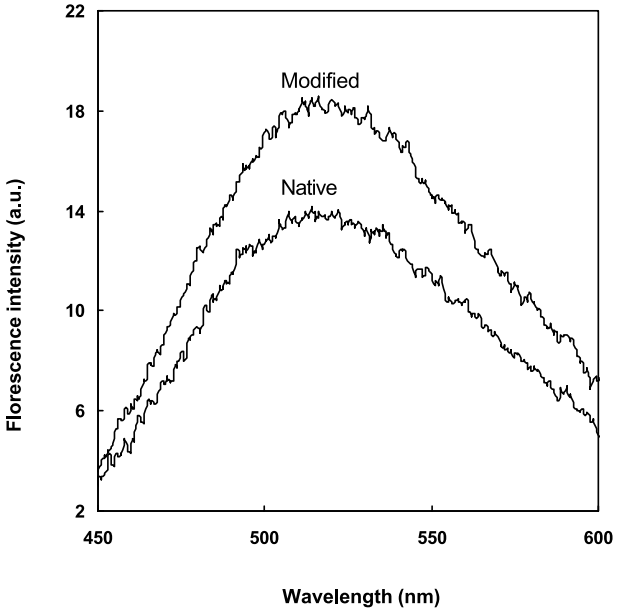


Fig. 6. Fluorescence spectra of 50 μM ANS in the presence of 50 μg/ml of native and modified glucose oxidase. For further details see Section 2.

References

[1] Pazur, J.P., Kleppe, K. and Cepure, A. (1965) Arch. Biochem. Biophys. 111, 351–357.
[2] O'Malley, J.J. and Weaver, J.L. (1972) Biochemistry 11, 3527–3532.
[3] Hecht, H.J., Kalisz, H.M., Hendle, J. and Schmid, R.D. (1993) J. Mol. Biol. 229, 153–172.
[4] Swoboda, B.E.P. (1969) Biochim. Biophys. Acta 175, 380–387.
[5] Tsuge, H., Natsuaki, O. and Ohashi, K. (1975) J. Biochem. 78, 835–843.
[6] Ohgushi, M. and Wada, A. (1983) FEBS Lett. 164, 21–24.
[7] Christensen, H. and Pain, R.H. (1991) Eur. Biophys. J. 19, 221.
[8] Fink, A.L. (1995) Methods Mol. Biol. 40, 343–360.
[9] Uversky, V.N. and Ptitsyn, O.B. (1996) J. Mol. Biol. 255, 215–228.
[10] Torchin, V.P., Maksimenko, A.V., Smirnov, V.N., Berzin, I.V., Klibanov, A.M. and Martinek, K. (1979) Biochim. Biophys. Acta 567, 1–11.
[11] Ryan, O., Smyth, M.R. and Fagain, C.O. (1996) Enzyme Microb. Technol. 19, 63–67.
[12] Dolginova, E.A., Roth, E., Silman, I. and Weiner, L.M. (1992) Biochemistry 31, 12248–12254.
[13] Hosseinkhani, S., Moosavi-Movahedi, A.A. and Nemat-Gorgani, M. (2003) Appl. Biochem. Biotechnol. 110, 165–174.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Hosseinkhani, S. and Nemat-Gorgani, M. (2003) Enzyme Microb. Technol. 32, 179–184.
[16] Semisotnov, G.V., Rodionova, N.A., Razgulyaev, O.I., Uversky, V.N., Gripas, A.F. and Gilmanshin, R.I. (1991) Biopolymers 31, 119–128.
[17] Semisotnov, G.V., Rodionova, N.A., Kutystenkov, P., Ebert, B., Blank, J. and Ptitsyn, O.B. (1987) FEBS Lett. 224, 9–13.
[18] Tsuge, H. and Mitsuda, H. (1974) J. Biochem. 75, 399–406.
[19] Tsuge, H. and Mitsuda, H. (1973) J. Biochem. 73, 199–206.
[20] Dixon, H.B.F. and Perham, R.N. (1968) Biochem. J. 109, 312–314.
[21] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[22] Fields, R. (1971) Biochem. J. 124, 581–590.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Protasevich, I., Ranjbar, B., Lobachov, V., Markov, A., Gilli, D., Briand, D., Lafitte, J. and Haiech, R. (1997) Biochemistry 36, 2017–2024.
[25] Kunda, S., Sunda, M. and Jagannadaham, M.V. (1999) Biochem. Biophys. Res. Commun. 264, 635–642.
[26] Permyakov, E.A. (1993) in: Luminescent Spectroscopy of Proteins, CRC Press, Boca Raton, FL.
[27] Xu, X., Liu, Q., Liu, Y. and Yongshu, X. (2002) J. Protein Chem. 21, 123–129.
[28] Boren, K., Andersson, P., Larsson, M. and Carlsson, U. (1999) Biochim. Biophys. Acta 1430, 111–118.
[29] Ptitsyn, O.B. (1992) in: Protein Folding (Creighton, T.E., Ed.), pp. 245–302, Freeman, New York.
[30] Rajaraman, K., Raman, B. and Rao, C.M. (1996) J. Biol. Chem. 271, 27595–27600.
[31] Hosseinkhani, S., Szittner, R., Nemat-Gorgani, M. and Meighen, E. (2003) Enzyme Microb. Technol. 32, 186–193.
[32] Matulis, D., Baumann, C.G., Bloomfield, V.A. and Lourien, R.E. (1999) Biopolymers 49, 451–458.