J. Biochem. 123, 445-449 (1998)

The Conformational Change Induced by FAD in Covalently Flavinylated 6-Hydroxy-D-Nicotine Oxidase Does Not Require (8α)FAD-(N_3)Histidyl Bond Formation[1]

Michaela Stoltz and Roderich Brandsch[2]

Institut für Biochemie und Molekularbiologie, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany

Received for publication, October 6, 1997

The contribution of (8α)-(N_3)histidyl bond formation to the conformation of covalently flavinylated proteins was investigated by trypsin treatment of wild type and mutant versions of a model enzyme, 6-hydroxy-D-nicotine oxidase (6-HDNO) of *Arthrobacter nicotinovorans*. In the absence of FAD, apo-6-HDNO exhibited a conformation exposing a protease accessible site. Holoenzyme formation through FAD-attachment to His_{71} induced a conformational change in the protein that shielded the trypsin recognition site. This conformational change, however, did not require FAD-histidyl bond formation since trypsin resistance was also exhibited by a 6-HDNO·Cys_{71} mutant protein which was unable to bind FAD covalently. Replacement of Arg_{67}, an amino acid residue supposed to be essential in flavinylation, by Ala rendered the protein protease sensitive as did replacement of Pro_{73} by Ala. These amino acids apparently play an essential role in stabilizing the native protein conformation. The inability to reach the native conformation also prevented FAD attachment, indicating that a specific conformation of the protein is a prerequisite for FAD-histidyl bond formation. Deletion of Phe_{448} and Arg_{449} from the 458 amino acid residues-containing enzyme resulted in complete protease sensitivity, demonstrating that flavinylation takes place posttranslationally.

Key words: flavoenzymes, site-directed mutagenesis, protein conformation.

Over twenty flavoenzymes are known to contain a cofactor covalently attached to the polypeptide chain (*1*). In 6-HDNO, an enzyme involved in nicotine degradation by *Arthrobacter nicotinovorans*, as well as in most covalently flavinylated enzymes, the FAD moiety is bound *via* an (8α)-isoalloxazine-(N_3)histidyl linkage to the polypeptide chain. A distinguishing feature of the primary structure of 6-HDNO is the absence of the amino acid fingerprint, GlyXGlyXXGly, characteristic of the FAD-binding domain of most flavoenzymes (*2*). In this respect, 6-HDNO shows similarities to other enzymes with covalently attached FAD like L-gulono-γ-lactone oxidase (*3*), berberine bridging enzyme, and mitomycin resistance protein (*4*). The FAD-binding domains of these enzymes may differ from the classical AMP-binding site of NAD- and FAD-dependent enzymes. In 6-HDNO the covalent attachment of FAD proceeds autocatalytically (*5*). Autoflavinylation depends on a flavinylation competent conformation and the bound cofactor stabilizes the conformation of the protein which becomes protease resistant (*6*). A mutant enzyme with noncovalently attached FAD produced by replacing His_{71} with Cys exhibited substrate turnover although with altered substrate kinetics (*7, 8*). Site-directed mutagenesis experiments demonstrated that attachment of FAD to His_{71} was prevented when Arg_{67} was replaced by Ala. This 6-HDNO mutant protein showed no enzymatic activity. However, introduction of lysine at position 67 of the 6-HDNO amino acid sequence restored the covalent FAD-binding of the protein and partially the enzyme activity (*9*). Therefore, a positively charged amino acid side chain at this position appeared to be essential for FAD attachment. Covalent FAD-binding was also abolished in a 6-HDNO polypeptide in which phenylalanine and arginine, positioned at 10 and 9 amino acid residues, respectively, from the carboxyterminus of the protein, were deleted (ΔFR, *10*).

The biochemical reason for the covalently bound FAD in these enzymes is not obvious, but FAD-histidyl bond formation might be required for the stabilization of the native conformations of the proteins. Here we challenged this assumption by examining the conformations of apo- and holo-wild type and mutant 6-HDNO by trypsin digestion.

MATERIALS AND METHODS

Materials—FAD, glycerol-3-P, trypsin, and soybean trypsin inhibitor were purchased from Sigma (Deisenhofen, FRG), phenylmethylsulfonylfluoride (PMSF) was from Roth (Karlsruhe, FRG), and [^{35}S]methionine ([^{35}S]-Met) was from Amersham (Braunschweig, FRG).

In Vitro Transcription and Translation Assays—Coupled transcription and translation in *Escherichia coli* S30 cell extracts in the presence of [^{35}S]Met were performed

[1] This research was funded by a grant from the *Deutsche Forschungsgemeinschaft* to R.B.

[2] To whom correspondence should be addressed. Tel: +49-761-203-5231, Fax: +49-761-203-5253, E-mail: brandsch@ruf.uni-freiburg.de

according to the suppliers instructions (Promega, Madison, USA). The assays were primed with 1 μg pLM-6-HDNO DNA carrying the 6-HDNO gene under the control of the tac-promoter. 6-HDNO holoenzyme formation was achieved by transcription and translation in the presence of 10 μM FAD and posttranslational incubation with 10 mM glycerol-3-P for 30 min at 30°C. The labeled translation products were analysed by SDS-PAGE. Densitometric quantification of the autoradiographs was performed with an Image Master DTS from Pharmacia (Freiburg, FRG).

Protease Treatment—The [^{35}S]Met labeled 6-HDNO protein synthesized in *E. coli* S30 cell extracts was incubated with 0.2 mg/ml trypsin for 15 min at 0°C. Digestion was stopped by the addition of 4 mg/ml soybean trypsin inhibitor. The proteins were submitted to SDS-PAGE.

Preparation of Cell Extracts and Determination of 6-HDNO Activity—*E. coli* JM109 cells transformed with pLM-6-HDNO carrying the wild type or mutant 6-HDNO gene were grown in LB medium (*11*) overnight at 30°C with agitation, and the cell extracts were prepared as described previously (*6*). The protein concentration of a cell extract was determined with the Bio-Rad protein assay (BioRad, Munich, FRG). 6-HDNO activity was determined photometrically (*12*). For holoenzyme formation, 100 μl of a cell extract was incubated with 10 μM FAD and 10 mM glycerol-3-P for 30 min at 30°C.

Site-Directed Mutagenesis—The replacement of proline codon 73, CCG, in the 6-HDNO gene by alanine codon, GCG, was performed with a mutagenic oligonucleotide as a 5′-primer for PCR with the 6-HDNO DNA as a template, as described previously (*10*). The PCR amplified 6-HDNO DNA fragment, flanked by restriction sites *Bgl*II and *Nsi*II, was used to replace the corresponding wild-type DNA in the 6-HDNO gene.

RESULTS AND DISCUSSION

Protease Susceptibility of the Apo- and Holoenzyme Conformations of the Wild Type Enzyme—The 6-HDNO polypeptides analysed in this work are presented in Fig. 1. We first examined the trypsin sensitivity of the wild type protein. To perform this 6-HDNO was translated in an *E. coli* S 30 *in vitro* transcription-translation system in the presence of [^{35}S]Met and then the labeled protein was treated for increasing times with trypsin (Fig. 2, panel A). Analysis of the digestion products by SDS-PAGE showed the processing of a part of the translated 6-HDNO into a faster migrating protein, approximately 3,000 Da lighter than the native protein. Both proteins were inaccessible to further protease attack over the time period examined (Fig. 2, panel A). Therefore, in subsequent experiments we performed 15 min trypsin digestion. A protein band migrating to the position of the 6-HDNO translation product following trypsin digestion apparently represented the protease-resistant holoenzyme. This assumption was tested directly, as presented in Fig. 2, panel B. Conversion of apo-6-HDNO into the holoenzyme requires, besides FAD, the presence of glycerol-3-P, or a related phosphorylated tricarbon compound (*5*). When *in vitro* 6-HDNO translation was performed in the presence of FAD, followed by incubation with glycerol-3-P and then the assay was treated with trypsin, an increase in the intensity of the protease resistant band migrating to the position of the native 6-HDNO (Fig. 2, panel B, □ and ■), and a decrease in the intensity of the processed 6-HDNO band (Fig. 2, panel B, ○ and ●) were observed. Densitometric evaluation of the autoradiograms supported the conclusion that the upper trypsin-resistant band represented the holoenzyme and the lower band represented the trypsin processed apoenzyme, since incubation with FAD and glycerol-3-P increased the amount of the trypsin-resistant protein present in the upper band with a parallel decrease in the amount of the protein species sensitive to trypsin (Fig. 2, panel C). Approximately 50% of the total amount of the 6-HDNO protein translated in the *E. coli in vitro* system was probably misfolded and completely degraded by the protease, 25% represented the trypsin-resistant holoenzyme and 25% represented the apoenzyme. The apoenzyme conformation must expose one or several trypsin recognition sites, since digestion with the protease removed an approximately 3,000 Da peptide fragment from an otherwise trypsin-resistant core. FAD incorporation during holoenzyme formation apparently induced a conformational change in the protein that made the previously exposed trypsin recognition site(s) inaccessible.

The results of protease treatment were correlated with enzyme activity determined in extracts of *E. coli* cells expressing wild type 6-HDNO (Fig. 2D, lane 1). Incubation of these cell extracts with only FAD did not lead to an increase in enzyme activity (Fig. 2D, lane 2) in agreement with the conclusion that the increase in trypsin resistance following incubation with FAD was due to a noncovalently bound cofactor. Generation of enzyme activity needed incubation of a cell extract with both FAD and glycerol-3-P (Fig. 2D, lane 3), which reflects the formation of the covalent FAD-histidyl bond (*5*).

amino acid replacements in the FAD-binding site of 6-HDNO			Reference
wt	$V_1...R_{67}S_{68}G_{69}G_{70}H_{71}N_{72}P_{73}...F_{448}R_{449}...S_{458}$ FAD	covalent	(12)
A1	A_{67}	noncovalent	(9)
C, S, Y	C_{71}, S_{71} or Y_{71}	noncovalent	(7)
A3	A_{73}	noncovalent	this work
ΔFR	$\Delta F_{448}R_{449}$	noncovalent	(10)

Fig. 1. **Amino acid replacements introduced by site-directed mutagenesis into the 6-HDNO protein.** Indicated is the position of the mutation in the 6-HDNO sequence and the modality of cofactor binding. The one letter code for amino acids is used.

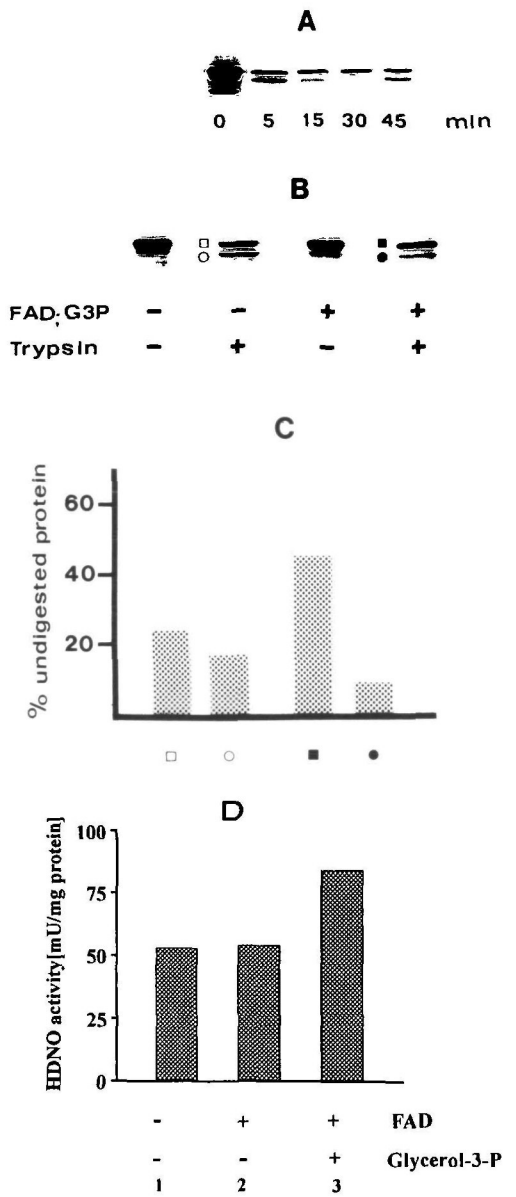

Fig. 2. **Trypsin treatment of the [^{35}S]Met labeled 6-HDNO wild type protein translated in the *E. coli* transcription-translation system and the 6-HDNO activity in *E. coli* cell extracts.** Transcription-translation assays primed with pLM-6-HDNO in the presence of [^{35}S]Met were performed as indicated under "MATERIALS AND METHODS." Equal aliquots were removed and treated with trypsin, and then the labeled protein was analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. Panel A, time course of trypsin digestion of 5 μl of [^{35}S]Met labeled 6-HDNO translation assay mixture at 0°C. The reactions were stopped at the time points indicated by the addition of trypsin inhibitor. Panel B, 5 μl aliquots of a 6-HDNO translation assay mixture (lane 1) or following holoenzyme formation (lane 3) were subjected to trypsin digestion and then analysed by SDS-PAGE. Panel C, densitometric quantification of the [^{35}S]Met labeled 6-HDNO protein bands generated on trypsin digestion expressed as a percentage of the undigested [^{35}S]Met labeled 6-HDNO protein present in an equal aliquot of the translation assay mixture. □ and ○ indicate the apoenzyme, ■ and ● the processed protein; G3P, glycerol-3-phosphate. Panel D, extracts were prepared from *E. coli* cells transformed with pLM-6-HDNO and grown at 30°C in LB medium overnight. Enzyme activity was determined in samples without any addition, following incubation for 30 min at room temperature with 10 μM FAD, or following incubation for 30 min at 30°C with 10 μM FAD and 10 mM glycerol-3-P (G3P), as indicated in the figure.

Protease Sensitivity of Mutant Proteins—Next we compared the trypsin digestion pattern of the wild type 6-HDNO protein with those of various mutant proteins (Fig. 3). The translation efficiency of the mutant proteins in the *E. coli in vitro* coupled transcription-translation system was similar to that of the wild type control (compare lanes 1, Fig. 3). Trypsin digestion of the translated proteins was performed with an identical aliquot of the same translation assay mixture in either the absence of FAD (compare lanes 1 and 2, Fig. 3) or with added FAD, followed by incubation with glycerol-3-P (compare lanes 3 and 4 in the panels of Fig. 3). Incubation with glycerol-3-P only had no effect on the 6-HDNO conformation (not shown). The mutant protein with His_{71} replaced by Cys, which does not bind FAD covalently, exhibited the same trypsin digestion pattern as the wild type protein (Fig. 3, panel C). The same result was obtained with the Ser_{71} and Tyr_{71} mutant proteins (7), which are both unable to bind FAD covalently (Fig. 3, panel S and panel Y, respectively). Thus, noncovalently bound FAD induced a similar conformational change in the mutant proteins to that observed in the wild type protein, shielding the trypsin recognition site from protease attack. When we analysed the conformation of the A1 mutant protein, where Arg_{67} was replaced by Ala, it became apparent that the protein had become trypsin-sensitive, including the trypsin-resistant core of the apoenzyme (Fig. 3, panel A1, lanes 1 and 2). Incubation with FAD and glycerol-3-P did not change the protease digestion pattern (Fig. 3, panel A1, lanes 3 and 4), and did not result in enzyme activity (not shown). These results suggest that a positively charged amino acid residue at this position is essential for the folding of the 6-HDNO protein into its native conformation. The close proximity of Pro_{73} to FAD-binding His_{71} and secondary structure considerations prompted us to exchange this proline residue with an alanine. Only a small amount of the mutant protein folded into the trypsin resistant core (Fig. 3, panel A3, lane 2). Translation in the presence of FAD and incubation with glycerol-3-P did not significantly increase the trypsin-resistant protein species (Fig. 3, panel A3, lane 4). The mutant protein exhibited only residual enzyme activity (less then 0.1% of wild type). Analysis by SDS-PAGE of the mutant protein immunoprecipitated with 6-HDNO specific antiserum from lysates of *E. coli* cells transformed with pLM-6-HDNO-Ala_{73} revealed the lack of FAD fluorescence of the protein, which was indicative of the absence of a covalently bound cofactor (data not shown). Thus, replacement of Pro_{73} with Ala impaired the folding of the 6-HDNO aminoterminal domain into the trypsin-resistant conformation of the holoenzyme. The 6-HDNO deletion mutant, ΔFR, missing Phe_{448} and Arg_{449}, exhibited no enzyme activity (data not shown), and was completely degraded by trypsin (Fig. 3, panel ΔFR).

Our results show that the formation of a FAD-histidyl bond was not involved in the conformational change induced in 6-HDNO on interaction of FAD with the protein. We assume that the conformational change took place in the FAD-binding domain of the protein. We infer this from the facts that it was induced by FAD and that formation of the protease-resistant core of the apoenzyme required the carboxyterminus, as demonstrated by the ΔFR mutant. We can not exclude the possibility that the trypsin cleavage site, which releases an approximately 3,000 Da peptide

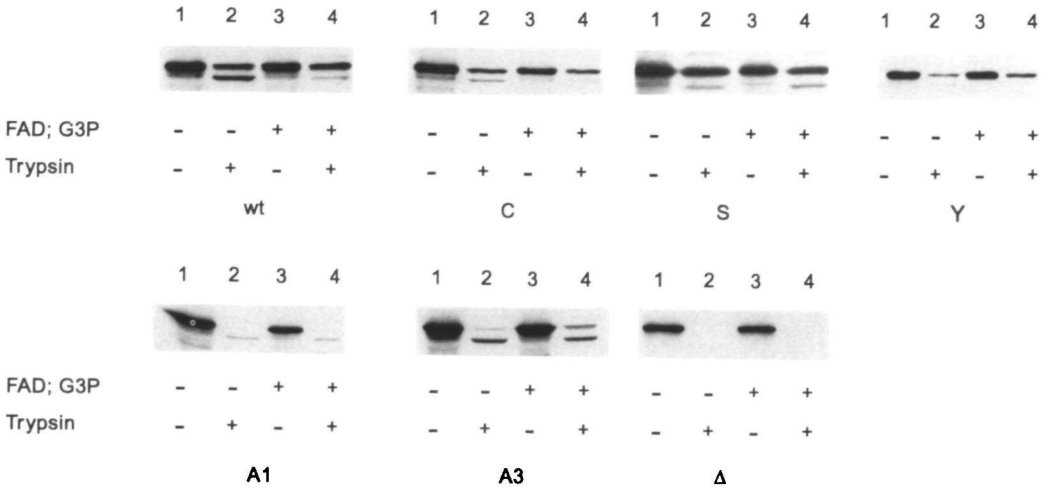

Fig. 3. **Analysis of the trypsin digestion pattern of the 6-HDNO wild type and mutant proteins.** 6-HDNO proteins were translated in the *E. coli* transcription-translation system as described in the legend to Fig. 2, in either the absence of any additions (lanes 1), or the presence of 10 μM FAD, followed by incubation with 10 mM glycerol-3-P (lanes 3). The translation assay mixtures were split into two, one half being subjected to trypsin digestion (lanes 2 and 4) for 15 min at 0°C. Following trypsin digestion, the [^{35}S]Met labeled proteins were separated by SDS-PAGE and the analysed by autoradiography. G3P, glycerol-3-phosphate; the abbreviations for the wild type (wt) and mutant proteins are as indicated in Fig. 1.

upon denaturation, is located in the carboxyterminal part of the protein, but that trypsin cleavage does not result in the release of the peptide under nondenaturing conditions. It has been shown for fumarate reductase (*13*), that replacement of FAD-binding His_{44} in the flavoprotein subunit (FrdA) by a Ser, Cys, or Tyr residue resulted in a reduced level of enzyme activity. A totally inactive enzyme complex was obtained on the replacement of His_{44} with Arg. However, the mutant enzyme complexes associated normally with the cytoplasmic membrane and contained stoichiometric amounts of noncovalently bound FAD. In accordance with our findings, the flavoprotein subunits with His_{44} replaced by other amino acid residues apparently could fold into a native-like conformation able to incorporate FAD noncovalently, and to associate correctly with the FrdB subunit and the cell membrane.

It appeared that a positively charged amino acid residue at position 67 of the protein was important for covalent FAD attachment (*9*). The data presented in this work show that Arg_{67} also plays an essential role in the folding and stabilization of 6-HDNO. The inability of the mutant protein to fold into the native conformation explains the lack of enzyme activity and covalent FAD attachment observed before. Since Arg_{67} is required for the folding of the trypsin-resistant core of the apoenzyme, the trypsin-sensitive site that disappears following holoenzyme formation could be located at an Arg or Lys residue preceding Arg_{67}, *e.g.* Arg_{34}, which would release a peptide fragment of 3,134 Da (*10*). Secondary structure predictions indicate that in enzymes with covalently bound FAD the modified His residue is located close to a β-turn (*14*). Therefore, replacement of Pro_{73} may disturb this structure and reduce the efficiency of holoenzyme formation. The trypsin sensitivity of the deletion mutant, 6-HDNO-ΔFR, positioned at the very carboxyterminal end of the protein makes cotranslational attachment of FAD to the nascent polypeptide chain unlikely. These amino acid residues are still covered by the translating ribosome before the release of the polypeptide chain. Therefore, it appears that the carboxyterminal amino acid residues are essential for the folding of the 6-HDNO protein and the attainment of a flavinylation competent conformation.

REFERENCES

1. Decker, K. (1991) Covalent flavoproteins in *Chemistry and Biochemistry of Flavoenzymes* (F. Müller, ed.) Vol. II, pp. 343-375, CRC Press, Boca Raton
2. Wierenga, R.K., Drenth, J., and Schulz, G. (1983) Comparison of the three-dimensional protein and nucleotide structure of the FAD-binding domain of *p*-hydroxybenzoate hydroxylase with the FAD as well as the NADPH-binding domains of glutathione reductase. *J. Mol. Biol.* **167**, 725-739
3. Koshizaka, T., Nishikimi, M., Ozawa, T., and Yagi, K. (1987) Isolation and sequence analysis of a complementary DNA encoding rat liver L-gulono-γ-lactone oxidase, a key enzyme for L-ascorbic acid biosynthesis. *J. Biol. Chem.* **263**, 1619-1621
4. August, P.R., Flickinger, M.C., and Sherman, D.H. (1994) Cloning and analysis of a locus (*mcr*) involved in mytomycin C resistance in *Streptomyces lavendulae*. *J. Bacteriol.* **176**, 4448-4454
5. Brandsch, R. and Bichler, V. (1991) Autoflavinylation of apo-6-hydroxy-D-nicotine oxidase. *J. Biol. Chem.* **266**, 19056-19062
6. Brandsch, R., Bichler, V., and Krauss, B. (1989) Binding of FAD to 6-hydroxy-D-nicotine oxidase apoenzyme prevents degradation of the holoenzyme. *Biochem. J.* **258**, 187-192
7. Mauch, L., Bichler, V., and Brandsch, R. (1989) Site-directed mutagenesis of the FAD-binding histidine of 6-hydroxy-D-nicotine oxidase. *FEBS Lett.* **257**, 86-88
8. Stoltz, M., Henninger, H.-P., and Brandsch, R. (1996) The design of an alternative, covalently flavinylated 6-hydroxy-D-nicotine oxidase by replacing the FAD-binding histidine by cysteine and reconstitution of the holoenzyme with 8-(methylsulfonyl)FAD. *FEBS Lett.* **386**, 194-196
9. Mauch, L., Bichler, V., and Brandsch, R. (1990) Lysine can replace arginine 67 in the mediation of covalent attachment of FAD to histidine 71 of 6-hydroxy-D-nicotine oxidase. *J. Biol. Chem.* **265**, 12761-12762
10. Brandsch, R., Bichler, V., Mauch, L., and Decker, K. (1993) Cysteine to serine replacements in 6-hydroxy-D-nicotine oxidase.

J. Biol. Chem. **268**, 12724-12729
11. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp. 504-506, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Brühmüller, M., Möhler, H., and Decker, K. (1972) Covalently bound flavin in D-6-hydroxynicotine oxidase from *Arthrobacter oxidans*. Purification and properties of D-6-hydroxynicotine oxidase. *Eur. J. Biochem.* **29**, 143-151
13. Blaut, M., Whittaker, K., Valdovinos, A., Ackrell, B.A.C., Gunsalus, R.P., and Cecchini, G. (1989) Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin. *J. Biol. Chem.* **264**, 13599-13604
14. Suzuki, H. and Kawamura-Konishi, Y. (1991) Cysteine residues in the active site of *Chorynebacterium* sarcosine oxidase. *J. Biochem.* **109**, 909-917