Effect of denaturants on the structure and activity of 3-hydroxybenzoate-6-hydroxylase

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Received 20 September 2005; revised 17 April 2006

The effect of denaturants such as urea, sodium dodecyl sulphate (SDS), guanidinium hydrochloride (Gu.HCl) on the structure of enzyme 3-hydroxybenzoate-6-hydroxylase was studied using intrinsic fluorescence and far and near-UV-CD spectroscopic techniques. Also, activity profiles of the enzyme, as a function of increasing concentrations of denaturants were studied. The far-UV CD spectrum of the enzyme did not show appreciable alterations in the presence of urea, SDS or Gu.HCl, thereby suggesting that the protein does not undergo gross conformational changes in its α-helical secondary structure. The treatment of enzyme with 2 M urea resulted in almost complete loss of catalytic activity, accompanied by the reduction of emission fluorescence of enzyme. Similarly, treatment with 0.01% SDS also caused almost complete loss of activity and quenching of enzyme fluorescence as well as a red shift in the emission peak. In addition, reduction in the intensity of near-UV-CD spectrum, especially at 280 nm was observed. About 70% of the activity was lost by treatment with 20 mM Gu.HCl, accompanied by quenching of intrinsic fluorescence of the enzyme. The change in intrinsic fluorescence of the enzyme in the presence of 5 mM-100 mM Gu.HCl could be correlated to progressive loss of catalytic activity. Thus, intrinsic fluorescence (due to tryptophan residues) could be used as an effective probe to provide an insight into the relation between the activity and subtle conformational changes of the enzyme. The results suggested that denaturants caused very slight conformational changes in the enzyme that perturbed the microenvironment of aromatic amino acid residues such as tryptophan accompanied by reduction or loss of catalytic activity.

Keywords: 3-Hydroxybenzoate-6-hydroxylase, Circular dichroism, Fluorescence, Denaturants, Guanidinium hydrochloride, Sodium dodecyl sulphate, Urea

The flavin containing enzyme 3-hydroxybenzoate-6-hydroxylase is an external flavin monooxygenase that is inducible in Pseudomonads1,2,3, Micrococcus sp.4 and Klebsiella pneumoniae5,6 by the presence of 3-hydroxybenzoate (3-HBA). The enzyme is unique, since it catalyzes para hydroxylation (unlike other monooxygenases which catalyze ortho hydroxylation) of 3-HBA to 3,6-dihydroxybenzoate (gentisate) and requires NADH or NADPH as the external reductant for its hydroxylating activity.

Earlier, we reported the facile purification of a NADH-dependant 3-HBA-6-hydroxylase from Micrococcus sp. to homogeneity by employing 3-HBA-dependent blue Sepharose affinity chromatography4. We also reported the involvement of arginine, histidine and tryptophan residues in catalytic functioning of the same enzyme through chemical modification studies7,8. Subsequently, we also characterized the interaction of enzyme with its aromatic substrate 3-HBA and the affinity ligand cibacron blue using kinetic and optical spectroscopy9,10. We observed partial inactivation of enzyme and diminished stability of the enzyme-substrate complex in response to treatment with 1 M urea9.

This paper reports the effect of denaturants such as urea, sodium dodecyl sulphate (SDS) and guanidinium hydrochloride (Gu.HCl) on activity and optical spectroscopic properties of the enzyme 3-HBA-6-hydroxylase. The objective of present study is to evaluate the sensitivity of catalytic activity to conformational changes and perturbations induced by denaturants in the enzyme.

Materials and Methods

Flavin adenine dinucelotide (FAD), NADH and urea were purchased from Sigma Chemical Co., USA

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Abbreviations used: CD, circular dichroism; FAD, flavin adenine dinucleotide; Gu.HCl, guanidinium hydrochloride; 3-HBA, 3-hydroxybenzoate; SDS, sodium dodecyl sulphate; TFE, 2,2,2-trifluoroethanol
earlier paper. Purified according to the methods described in our earlier paper. 3-HBA-6-hydroxylase was purified according to the methods described in our earlier paper. The other chemicals used were of analytical reagent grade and locally available. 3-HBA-6-hydroxylase was purified according to the methods described in our earlier paper. Buffer-A was 50 mM KH2PO4-NaOH buffer, pH 8.2.

**Effect of denaturants on activity of the enzyme**

Aliquots of enzyme (2 µg/ml) in buffer-A were incubated separately with urea, SDS and Gu.HCl for 5-10 min, prior to measuring the enzyme activity. The concentrations of urea and Gu.HCl ranged from 0.1 M to 6 M and 5 mM to 100 mM, respectively, while those of SDS were 0.001% and 0.01%. Polarographic activity measurements were done as described in our earlier paper. Fluorimetric measurements were carried out using a Hitachi 650 fluorescence spectrophotometer in the ratio mode using slit widths of 5 nm for excitation and emission. The fluorimetric assay of the enzyme was conducted by following increase in the intensity of emission fluorescence at 440 nm (excitation at 335 nm) as a function of time, which resulted from the accumulation of hydroxylated product, gentisate.

**Effect of denaturants on fluorescence properties of the enzyme**

**Treatment with urea**

The enzyme (25 µg/ml) in buffer-A was incubated with 2 M urea for 24 h at 30°C to achieve complete denaturation. The intrinsic fluorescence emission spectrum of the denatured enzyme was monitored (λexc = 295 nm) after subtracting from blank solution containing 2 M urea in buffer-A. In order to study the effect of urea on the flavin fluorescence spectral property, the enzyme (25 µg/ml) in buffer-A was denatured in 3 M urea for 24 h at 30°C. The spectrum was recorded after subtracting from blank solution containing 3 M urea. The absorbance of 2 M urea solution at 295 nm was less than 0.1 and, therefore, the inner filter effect can be neglected.

**Treatment with Gu.HCl and SDS**

The enzyme (25 µg/ml) in buffer-A was treated with different concentrations of Gu.HCl (3, 8 and 138 mM) or SDS (0.01%) and pre-incubated for 10 min, before recording the fluorescence emission spectra (λexc = 285 nm and 295 nm for Gu.HCl and SDS-treated enzyme, respectively).

**Effect of denaturants on CD spectral properties of the enzyme**

Aliquots of 1 ml buffer-A containing enzyme were treated separately with urea (2 M), Gu.HCl (100 mM) and SDS (0.01%) for 24 h. The far-UV and near-UV-CD spectra of the denatured enzyme were compared with the native enzyme. The concentrations of enzyme used were 50 and 75 µg/ml for the far and near-UV-CD regions, respectively.

**Results**

**Effect of denaturants on activity of the enzyme**

In general, there was a progressive loss in catalytic activity of 3-HBA-6-hydroxylase with increasing concentrations of denaturants, namely urea, SDS and Gu.HCl. The extent of loss of enzyme activity following treatment with 2 M urea, 0.01% SDS and 100 mM Gu.HCl were 90, 98 and 95%, respectively.

**Effect of urea**

The progressive inactivation of the enzyme as a function of the concentration of urea as measured by polarographic and fluorimetric assay methods is depicted in Figs 1A and 1B, respectively. It may be noted that two inactivation curves obtained by monitoring the rates of disappearance of oxygen and appearance of the product gentisate, overlap on each other fairly well, thereby confirming the reliability of two assay methods used.

**Effect of SDS**

The addition of SDS in reaction mixture resulted in almost total inhibition of oxygen consumption (measured by polarographic assay) as well as the formation of gentisate (measured by emission fluorescence), thereby confirming the correlation between two assay methods used. We were unable to obtain a profile of concentration-dependant loss of enzyme activity, since SDS even at concentrations as low as 0.01% led to complete loss of activity.

**Effect of Gu.HCl**

The treatment of 3-HBA-6-hydroxylase with increasing concentrations of Gu.HCl resulted in the gradual decline in the catalytic activity (Fig. 1C), as monitored by polarographic assay. It is evident from the figure that the activity of the enzyme is very sensitive to the presence of denaturant since ~70% of the activity is lost, even with addition of 20 mM Gu.HCl.

**Effect of denaturants on fluorescence properties of the enzyme**

**Effect of urea**

The fluorescence emission spectra of the native and urea-denatured 3-HBA-6-hydroxylase are compared...
Although the emission spectrum of urea-denatured enzyme does not undergo shift, there is considerable reduction in the intensity of emission fluorescence intensity, as compared to that of native enzyme. The absorbance of 2 M urea solution at 295 nm ($\lambda_{exc}$) was less than 0.1 and, therefore, the inner filter effect is insignificant. Reduction in the intensity of emission fluorescence may be related to the reduced hydrophobicity of denatured protein in comparison to native enzyme. X-ray crystallographic studies of diketopiperazine-urea cocrystal revealed hydrogen bonding between amide groups and urea. It was also demonstrated that urea altered the exchange rate of surface Arg and/or Lys protons in bovine pancreatic trypsin inhibitor.
Effect of SDS

The effect of SDS on intrinsic emission fluorescence spectrum of enzyme is shown in Fig. 2B. There is a red shift of 10 nm that is accompanied by marginal decrease in the intensity of emission fluorescence of SDS-treated enzyme. The absorbance of 0.01% SDS solution at 295 nm ($\lambda_{exc}$) was less than 0.1 and, therefore, the inner filter effect is insignificant.

Effect of Gu.HCl

The effect of increasing concentrations of Gu.HCl on the emission fluorescence spectrum of enzyme is shown in Fig. 2C. The figure reveals blue shift of 5 nm associated with gradual quenching of the emission fluorescence with increasing concentration of the denaturant. Blank solutions containing corresponding concentrations of Gu.HCl had absorbance values less than 0.1 at 285 nm ($\lambda_{exc}$) and, therefore, the inner filter effect is insignificant. The incremental reduction in fluorescence intensity of the enzyme at 335 nm, (the emission peak of native enzyme) in the presence of increasing concentrations of Gu.HCl could be linked to the progressive loss of activity of the enzyme. This is depicted in Fig. 3 and superimposition of points on a single curve is significant, as it suggests a negative correlation between the conformational changes of enzyme and its catalytic activity.

Effect of urea and SDS on flavin fluorescence spectrum of the enzyme

The inactivation of enzyme by 3 M urea or 0.01% SDS resulted in about 20% enhancement of flavin fluorescence ($\lambda_{exc} = 450$ nm) of enzyme (spectra not shown). Results obtained by us suggest that catalytic activity of the enzyme is more sensitive to denaturation by the above-mentioned agents, as compared to the microenvironment of FAD moiety of protein.

Effect of denaturants on CD spectral properties of the enzyme

Effect of urea

The far-UV-CD spectrum (figure not shown) and near-UV-CD spectrum (Fig. 4A) of urea-denatured enzyme show insignificant changes, as compared to native enzyme. Thus, the enzyme does not seem to undergo detectable conformational changes in the backbone structure.

Effect of SDS

The far-UV-CD spectrum of the enzyme is not appreciably altered in the presence of SDS, indicating that the enzyme does not undergo gross conformational changes in the $\alpha$-helical secondary structure (spectrum not shown). On the other hand, the near-UV-CD spectrum (Fig. 4B) contributed by the $\pi-\pi^*$ transitions of side chains of aromatic amino acid residues undergoes major perturbations in the microenvironment of the active site.
Effect of Gu.HCl

The far-UV (Fig. 5) and near-UV-CD spectra (not shown) of the 100 mM Gu.HCl-treated enzyme are almost similar to those of native enzyme. Such observations in combination with activity profile (Fig. 1C) suggest loss of catalytic activity in the absence of appreciable changes in the gross conformation of the enzyme. Gu.HCl has been shown to be located near amino acid residues such as glutamine, arginine, serine, lysine etc. of proteins although there is no preference for any secondary structure with respect to the binding of denaturant to proteins\(^{13}\).

Discussion

The effect of denaturants upon the activity and optical spectroscopic properties of mono-oxygenases such as 3-HBA-6-hydroxylase is an important aspect to study, since it is well-known that the activity of these inducible enzymes is strictly related to their conformational state (mostly controlled by the presence or absence of the aromatic substrate undergoing hydroxylation). The interaction of denaturants with proteins may be direct\(^{14}\) or their high concentrations may change the behaviour of solvent\(^{15}\). Denaturants act as cross-linking agents and cause overall reduction in the conformational freedom of folded protein. Denaturants, such as urea and Gu.HCl unfold proteins by migrating into their interior regions and forming hydrogen bonds in the backbone or side chain atoms\(^{13,16}\). Other models suggest that denaturants decrease hydrophobic effect, thereby destabilizing protein structure\(^{17,18}\).

It is interesting to note that the presence of denaturants such as urea, SDS and Gu.HCl did not alter the far-UV-CD spectrum (backbone structure) of 3-HBA-6-hydroxylase significantly in the present study. Earlier\(^{19}\), it was reported that the far-UV-CD spectrum of hen white lysozyme remains unperturbed in 15% 2,2,2-trifluoroethanol (TFE), however, at concentration of TFE exceeding 15%, a substantial increase in the ellipticity at 222 nm was observed. Maithal et al\(^{20}\) studied wild type and mutated (Tyr74 substituted with glycine) triosephosphate isomerase from \textit{Plasmodium falciparum} using CD spectroscopy. The wild type enzyme was robust and did not show substantial loss in secondary structure, even after treatment with 8 M urea. In contrast, the secondary structure of mutated protein was disrupted by treatment with 2.6 M urea, thereby suggesting that the tyrosine 74 is a critical aromatic residue at subunit interface for stabilizing the protein structure.

The presence of denaturants led to change in the intensity and/or nature of intrinsic emission fluorescence spectrum (predominantly contributed by tryptophan residues), accompanied by loss of activity of 3-HBA-6-hydroxylase. For example, treatment with 0.01% SDS resulted in quenching of the enzyme fluorescence as well as a red shift in emission peak. Similarly, treatment with 1 M urea resulted in the reduction of emission fluorescence of enzyme. Such observations have also been reported earlier for D-glyceraldehyde-3-phosphate dehydrogenase and creatine kinase in the presence of denaturants\(^{21,22}\).

The change in intrinsic fluorescence of 3-HBA 6-hydroxylase in the presence of increasing concentrations of Gu.HCl could be correlated to progressive loss of catalytic activity. Similar observations have also been reported for Gu.HCl-induced denaturation of D-glyceraldehyde-3-phosphate dehydrogenase\(^{21}\). Thus, intrinsic fluorescence of tryptophan residues could be used as an effective probe to provide an insight into the relation between the activity and subtle conformational changes of the enzyme.

Yet another useful probe, which is sensitive to changes in the tryptophan environment, is the near-UV-CD spectrum of the protein. This technique has been exploited earlier to study the conformations of a protein in its native, denatured and modified states or in the presence of ligands\(^{23-29}\). The treatment of
3-HBA-6-hydroxylase with 0.01% SDS resulted in the reduction in the intensity of near-UV-CD spectrum, especially at 280 nm with concomitant loss of activity. These results suggest that stable orientation of aromatic side chains in tryptophan residues might be necessary for the activity of 3-HBA-6-hydroxylase. In summary, the results of the present study on the activity, fluorescence and CD (near-UV) properties of the enzyme, in the presence of denaturants point out subtle conformational changes of 3-HBA-6-hydroxylase that perturbs the microenvironment of aromatic amino acid residues such as tryptophan. Such changes are accompanied by reduction or loss of catalytic activity of the enzyme.

Acknowledgement

The financial support given by the Indian Institute of Science, Bangalore, India is greatly appreciated. We also acknowledge S L Nagraj for his excellent technical assistance.

References