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Effect of covalent attachment of neomycin on conformational and aggregation properties of catalase

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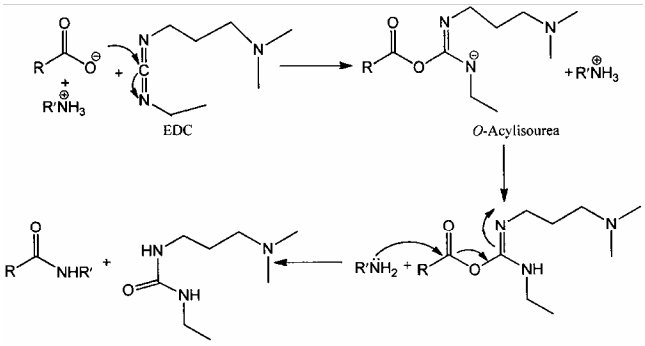
The carboxylic groups of glutamic acid and aspartic acid residues of catalase (CAT) were chemically modified using the treatment of the enzyme with 1-ethyl-3-(3'-dimethylamino) carbodiimide hydrochloride (EDC) and neomycin. The effect of covalent attachment of neomycin on the enzymatic activity, conformational and aggregation properties of CAT was investigated. The modification of CAT with different concentrations of neomycin showed two different types of behavior, depending up on the concentration range of neomycin. In the concentration range from 0.0 to 5.2 mM, neomycin-modified CAT, compared to the native enzyme exhibited higher α-helix content, reduced surface hydrophobicity, little enhancement in CAT activity and a better protection against thermal aggregation, whereas at concentrations greater than 5.2 mM, the modified enzyme exhibited a significant decrease in CAT activity and an increase in random coil content which may result in disorder in the protein structure and increase in thermal aggregation. This modification is a rapid and simple approach to investigate the role of aspartate and glutamate residues in the structure, function and folding of CAT.

Keywords: Aminoglycoside, Catalase, Chemical modification, Neomycin, Thermal aggregation

Chemical modification is one of the most practical methods to study protein structure-function relationships[1-3]. Generally, modifications are performed on the most reactive side chains and are predominantly oxidation, reduction, and nucleophilic and electrophilic substitutions. The carboxylic acid side chains in proteins have been modified by a number of different reagents, namely trialkyl oxonium fluoroborate salts, isoxazolium salts and carbodiimides[4-10]. Carbodiimides can react with carboxyl groups to yield an activated intermediate, most likely an acylisourea, which then reacts with a nucleophile, such as an amine to yield the desired amide and the urea by-product[11-13]. The reaction of an amino compound with a carboxyl group is outlined in **Scheme 1**.

Aminoglycosides constitute a large family of antibiotic molecules that are effective against aerobic gram-negative bacteria. They include streptomycin, kanamycin, neomycin, gentamicin, tobramycin, amikacin and netilmicin[14]. Also, due to their large positive charge density, the interaction of these drugs with negatively charged membrane phospholipids has been well-characterized[15-17]. Aminoglycosides are characterized by two or more amino sugars attached in glycosidic links to an aminocyclitol ring. They are water soluble and highly polar thermally labile compounds, however, the presence of primary amino groups in their structure suggests that such molecules might react with carboxylic acid groups of aspartic acid and glutamic acid residues of proteins.



Scheme 1—One-pot carbodiimide coupling

Neomycin B **(Scheme 2**) is a highly polar polycationic structure at physiological pH. The

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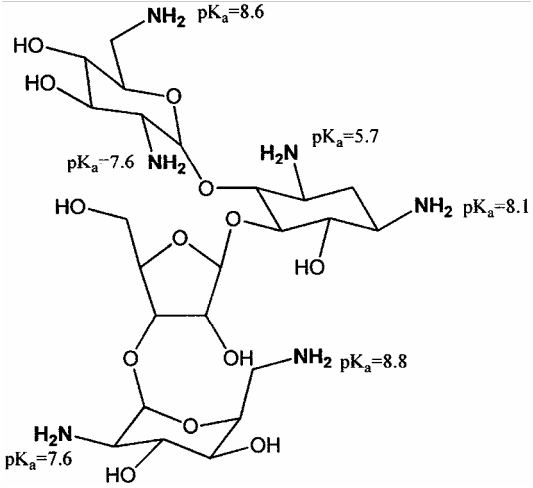

Scheme 2—Structure/pK_as of neomycin

number of positive charges carried by this compound is proportional to the number of its ionizable amino groups[18]. Although many studies have been reported to establish the interaction between protonated amino groups of neomycin and anionic molecules[15-20], but to the best of our knowledge, studies are lacking on the covalent binding between the amino groups and carboxyl residues of proteins.

Here, using neomycin as (an aminoglycoside compound with high content of amino groups) and 1-ethyl-3-(3'-dimethylamino) carbodiimide hydrochloride (EDC), we have investigated modification of free carboxyl groups of aspartic and glutamic acid residues of bovine liver CAT as a protein model. Catalase (H_2O_2: H_2O_2 oxidoreductase EC 1.11.1.6) exists as a dumb bell shaped tetramer of four identical subunits, each over 500 amino acids long. It contains four porphyrin heme groups that allow the enzyme to react with the hydrogen peroxide (H_2O_2)[21]. We have examined the covalent binding effects of neomycin on the enzymatic activity, thermal aggregation and structural properties at tertiary and secondary folding levels using spectroscopic methods, based on ultraviolet-visible (UV-vis), Fourier-Transform infrared (FT-IR), fluorescence and circular dichroism (CD) techniques.

Materials and Methods

Bovine liver catalase, neomycin B sulfate and 1-anilinonaphthalene-8-sulfonic acid (ANS) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). 1-Ethyl-3-(3'-dimethylamino) carbodiimide hydrochloride (EDC) and all other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). All reactions were carried out at room temperature.

Preparation of catalase solution

An appropriate amount of CAT suspension was dissolved in the 50.0 mM phosphate buffer (pH 7.0) and dialysed against buffer sufficiently, in order to remove the thymol preservative. Then, the concentration of the enzyme solution was determined by measuring its absorbance at 405 nm using 3.24×10^5 M^{-1} cm^{-1} for the molar extinction coefficient[22] and 250,000 Da for the molecular mass of bovine liver CAT.

Chemical modification of CAT

Selective modification of carboxyl groups of CAT was carried out with neomycin. CAT (0.5 mg/mL) in 50.0 mM phosphate buffer (pH 7.0) was mixed with EDC (0.0-156.0 mM) and incubated with neomycin (0.0–156.0 mM) for 1 h at room temperature. In all experiments, the ratio of EDC to neomycin was 1:1. In order to remove excess EDC and neomycin, the reaction mixtures were dialysed for three steps of 8 h each against 1000 volumes of phosphate buffer solution. Carbodiimide reacts with carboxyls to give an intermediate ***O***-acylisourea. Since the intermediate undergoes hydrolysis in aqueous solutions, we used a large excess of EDC than the enzyme, in order to compensate the hydrolysis of the intermediate at pH 7.0.

CAT activity assay

The activity of the enzyme was measured by following the rate of H_2O_2 decomposition at 240 nm. The reaction mixture contained phosphate buffer (pH 7.0) and 10.0 mM H_2O_2. The difference in absorbance (A_{240}) for unit time was a measure of the CAT activity[23].

Fourier-Transform infrared (FT-IR) spectroscopy

The modification of CAT with neomycin was confirmed by FT-IR analysis. FT-IR spectra were recorded using a JASCO (Tokyo, Japan) Fourier-Transform infrared instrument with KBr pellets.

Electrophoresis

The native enzyme and its modified forms were examined by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) mini gel (8.3 cm × 7.3 cm) and visualized by staining with Coomassie brilliant blue[24]. The molecular mass markers used were: phosphorylase B (98.0 kDa), BSA (62.0 kDa), glutamic dehydrogenate (49.0 kDa) and alcohol dehydrogenas (38.0 kDa).

Absorption spectroscopy

Absorption spectra of native and modified CAT in the wavelength range 200–800 nm, as well as determination of CAT concentration were performed using an Analytik Jena SPECORD 250 (Jena, Germany) spectrophotometer, equipped with a peltier temperature controller. The "Kinetic mode" of the spectrophotometer was also used to measure the rate of H_2O_2 decomposition by the enzyme.

Intrinsic fluorescence spectroscopy

Emission spectra of native and modified CAT (0.2 mg/mL) in the wavelength range 300-500 nm were recorded using a JASCO (Tokyo, Japan) spectrofluorometer. An excitation wavelength of 295 nm, slit widths of 10 nm and scan speed of 120 nm/min were used in all measurements.

ANS-fluorescence spectroscopy

The native or modified enzyme (0.075 mg/mL) was incubated with 9.0 µM ANS in 50.0 mM phosphate buffer (pH 7.0) for 15 min. The fluorescence emission of the complex was recorded over the range of 400-550 nm using an excitation wavelength of 380 nm.

Circular dichroism (CD) spectroscopy

Far-UV CD spectra of 0.2 mg/mL of the native and modified CAT in phosphate buffer (pH 7.0) in the wavelength range 190–260 nm with a spectral resolution of 1 nm, scan speed of 20 nm/min and a band width of 1 nm were recorded using a circular dichroism spectrometer model 215 (Aviv Instruments Inc. Lakewood, NJ, USA). Quartz cells with an optical path of 0.1 cm were used and all measurements were done at 25°C. All far-UV CD spectra were base line corrected by subtracting phosphate buffer spectrum and analyzed for the secondary structural elements of the native and modified enzyme using a cdnn Program Version 2[1]

Aggregation assays

Thermal aggregation of native and modified enzyme solutions (0.5 mg/mL) was followed at 360 nm in an Analytik Jena SPECORD 250 (Jena, Germany) spectrophotometer. Incubations were carried out at 55°C for 50 min.

Results and Discussion

FT-IR analysis

Although the UV-vis spectroscopy is a common technique for verifying the chemical modification of proteins, a direct UV-vis detection of covalent attachment of neomycin to CAT cannot be easily performed, due to the lack of a strong UV-vis absorbing chromophore in neomycin. Therefore, in this work, the modification of CAT with neomycin was established by FT-IR measurements. As shown in Fig. 1, spectra **a** and **b**, three amide bands (I, II and III), within ranges of 1700-1600 cm^{-1}, 1600-1500 cm^{-1} and 1200-1100 cm^{-1} were observed for both CAT and neomycin-modified CAT. The amide I band is caused by C=O stretching vibrations of peptide groups in the enzyme backbone. The amide II and III bands are attributed to a combination of N-H in-plane bending and C-N stretching of the peptide groups[25,26].

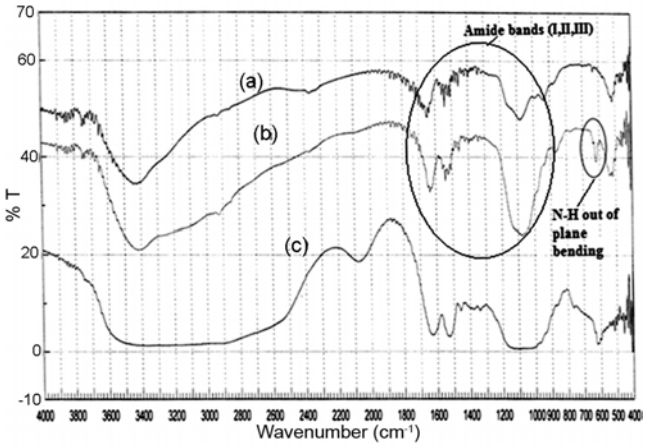

Fig. 1—FT-IR spectra of the native CAT (a), neomycin-modified CAT (b) and neomycin (c)

The FT-IR spectrum of neomycin (Fig. 1, spectrum **c**) showed bands around 1630, 1550 and 1100 cm^{-1} corresponding to the N-H bending, C-N stretching and ether frequencies, respectively[27] having overlap with amide bands of the enzyme. Furthermore, a band near 670 cm^{-1}, which was not observed in the FT-IR spectrum of the enzyme might be assigned to the N-H out of plane bending of neomycin. The presence of this band in the FT-IR spectrum of neomycin-modified CAT (spectrum **b**) might be due to the covalent attachment of neomycin to the enzyme.

Electrophoresis

The homogeneity of neomycin-modified CAT was evaluated by SDS-PAGE as shown in Fig. 2. Both unmodified and modified enzymes showed a single band between 62 and 48 kDa for each protein, demonstrating the homogeneity of the samples. It was noteworthy that the covalent attachment of neomycin to the enzyme resulted in a small increase in the apparent molecular mass of the modified forms of the enzyme (Fig. 2, Lanes 1 and 2).

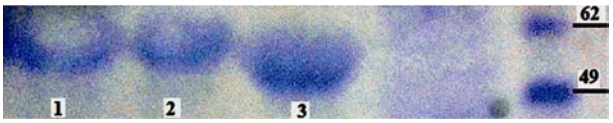

Fig. 2—SDS-PAGE of the unmodified and modified CAT [Lane 1**,** CAT + neomycin (5.2 mM); lane 2**,** CAT + neomycin (7.8 mM); and lane 3, unmodified CAT. Numbers on the right are molecular masses of the markers]

Absorption spectra

The UV–vis absorbance spectra of both native and neomycin-modified enzymes are shown in Fig. 3A. As can be seen, the absorption spectrum of the native enzyme exhibited maxima at about 278, 405 and 625 nm, which were attributed to the $\pi \rightarrow \pi^*$ transitions in aromatic amino acids, $\pi \rightarrow \pi^*$ transitions in the heme system and high-spin charge-transfer porphyrin ($p\pi$) to iron ($d\pi$), respectively . The absorption at about 405 nm is an intrinsic probe of CAT and is suitable for monitoring the interaction of the heme group with the protein moiety[21, 22]. In the presence of neomycin (0.0-156.0 mM) as modifier, a complex dependence of soret absorption (at 405 nm) of CAT was observed with increasing concentration of neomycin. As seen in Fig. 3A and B in the modifier concentration range 0.0-5.2 mM, the absorption at 405 and 625 nm increased (without any significant change in the absorption at 278 nm) with increasing concentration of neomycin, whereas in the concentration range 5.2-156.0 mM, the absorption at 405, 625 and 278 nm decreased with increasing concentration of neomycin, suggesting that neomycin had significant effect on the protein moiety in two different ways. The observed changes in the absorption spectrum of the enzyme in the modifier concentration range 0.0-5.2 mM might be due to increase in the number of ferric heme iron high-spin states of the enzyme, which can cause the increase in absorbance at 405 and 625 nm with no considerable change in absorbance at 278 nm. Whereas at concentrations higher than 5.2 mM, it seemed the decrease in absorption maxima (278, 405, 625 nm) was due to change in conformation of the protein and consequently change in heme pocket microenvironment.

Another sensitive probe can be regarded for observing very small conformational structure changes of neomycin-modified CAT is the enzymatic activity. Fig. 3C shows the changes in enzymatic activity of CAT as a function of neomycin concentration at room temperature. As can be seen, an initial slight enhancement in enzymatic activity

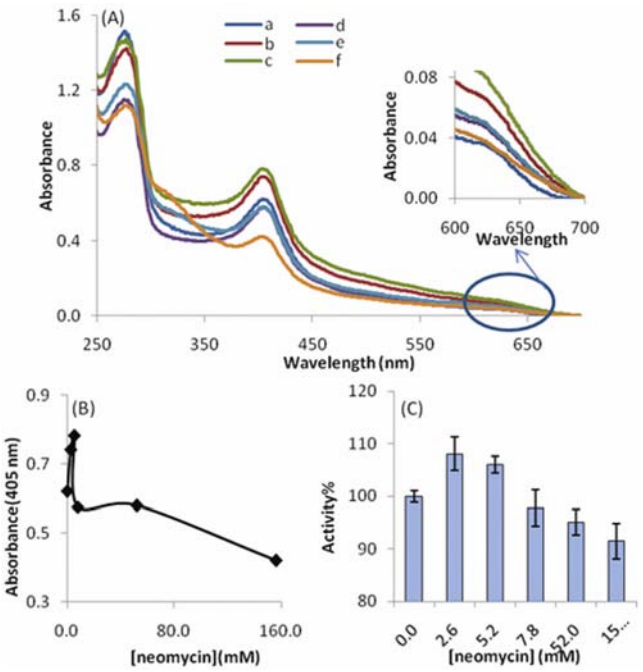

Fig. 3—(**A**): UV–vis absorbance spectra of the native CAT (a) modified CAT with neomycin concentrations of 2.6 mM (b), 5.2 mM (c), 7.8 mM (d), 52.0 mM (e), 156.0 mM (f) [The protein concentration was 0.5 mg/mL at pH 7.0. The inset shows UV-vis absorbance spectra of the native and modified enzyme over the range of 600-700 nm; (**B**) The plot of absorbance at 405 nm against neomycin concentration; and (**C**) The plot of residual activity percent against neomycin concentration]

(maximum ~8 %) compared to the native enzyme was observed between 0.0 and 5.2 mM neomycin. However, for neomycin concentrations above 5.2 mM, ther was a gradual decrease in enzymatic activity. Thus, this observation also confirmed the conformational changes in modified CAT, which in turn can be caused by the modification of carboxyl residues.

Fluorescence spectra

To obtain information on possible conformational changes at tertiary structural level that could occur as a result of the CAT modification by neomycin, intrinsic (tryptophan) fluorescence spectra of the native and modified enzyme with different concentrations of neomycin were recorded. As can be seen in Fig. 4A, at concentrations of neomycin up to 5.2 mM, a slight enhancement in tryptophan emission intensity (336 nm) of CAT without any shift in wavelength maximum was observed, while above this concentration a gradual decrease in tryptophan emission intensity was seen. From these results, it could be inferred that tryptophan residues of CAT

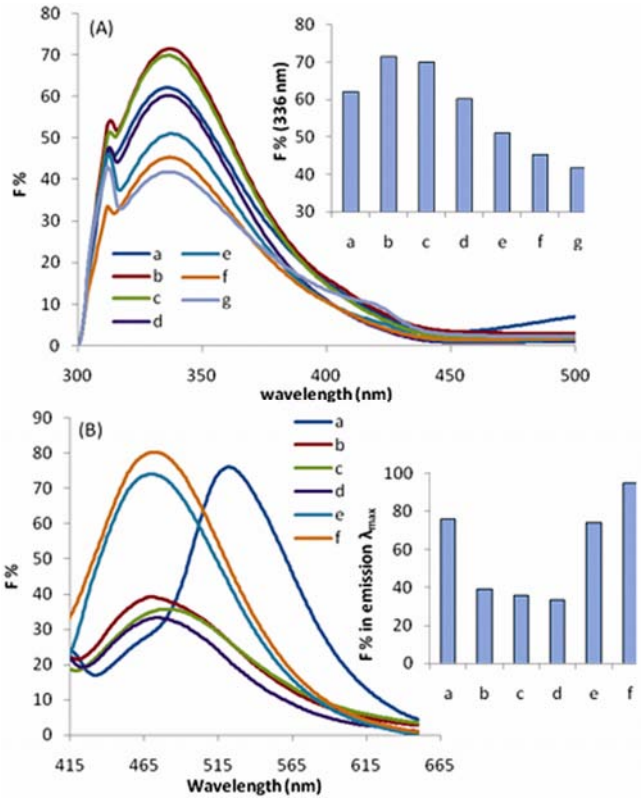

Fig. 4—(**A**): Intrinsic fluorescence spectra of native CAT (a) modified CAT with neomycin concentrations 2.6 mM (b), 5.2 mM (c), 7.8 mM (d), 30.0 mM (e), 52.0 mM (f), 156.0 mM (g) [The inset shows the fluorescence intensity at 336 nm against neomycin concentration. Experimental conditions: fluorescence measurements were carried out with 0.2 mg/mL of CAT in phosphate buffer (50 mM, pH 7.0). The excitation wavelength was 295 nm using a slit width of 10 nm]; (**B**) ANS fluorescence spectra in the presence of native CAT (a) and modified CAT with neomycin concentrations -2.6 mM (b), 5.2 mM (c), 7.8 mM (d), 52.0 mM (e), 156.0 mM (f). ANS fluorescence intensity at λ_{max} against neomycin concentration. Experimental conditions: ANS fluorescence measurements were carried out with 0.075 mg/mL of CAT in phosphate buffer (50 mM, pH 7.0). The excitation wavelength was 380 nm using a slit width of 10 nm]

which are buried in the interior of the enzyme molecule were affected by neomycin.

Furthermore, the fluorescence emission of ANS bound to native and modified CAT was used to compare their surface hydrophobicity. This fluorescent probe binds to solvent-accessible clusters of non-polar groups in proteins[28]. Figure 4B represents the fluorescence spectra of ANS bound to the native and modified CAT. The fluorescence emission of ANS in the presence of the native enzyme showed a significant intensity with the emission λ_{max} at about 523 nm, indicating the hydrophobic interaction between ANS and the exposed hydrophobic patches on the native CAT[29]. However, the fluorescence properties of ANS bound to the modified enzyme at different concentrations of neomycin showed a shift in emission λ_{max} from about 523 nm to about 473 nm along with a sharp quenching of ANS binding fluorescence for modified CAT after modification with neomycin in the range of 0.0 to 5.2 mM, indicating the reduction of exposed hydrophobic patches on the modification of the enzyme in this concentration range. At neomycin concentrations above 7.8 mM, there was a significant increase in ANS binding fluorescence for modified CAT. The significant increase in ANS fluorescence might be due to conformational changes in the protein in a way that make the more buried hydrophobic clusters to expose the solvent or might be due to the electrostatic interactions between the ANS-sulfonate groups and the positive charges of neomycin in this concentration range.

CD Spectroscopy

CD spectroscopy was used to investigate the changes in the structural properties of the protein at a secondary folding level, resulting in the chemical modification of the carboxylic groups of aspartic acid and glutamic acid residues. The far-UV CD spectra of the native and modified enzyme with different concentrations of neomycin in the range of (0.0-156.0 mM) are shown in Fig. 5. All spectra displayed a comparable shape with negative extremes in 209 nm and 222 nm (the latter more intense). Each spectrum analyzed for the contents of secondary structure elements using the cdnn Program Version 2. As seen in the inset of Fig. 5, compared to the native enzyme, the modification of CAT with neomycin up to 5.2 mM induced an increase of about 10% in α-helix content with a concomitant decrease in the amount of random coil, suggesting a more compact structure for neomycin-modified enzyme, whereas at neomycin concentrations greater than 5.2 mM, the random coil secondary structure was more predominant than α-helix structure, suggesting an increase in disordered structure.

Effect of chemical modification on thermal aggregation

As shown in Fig. 6, compared to the native enzyme, the modification of CAT with neomycin up to 5.2 mM effectively inhibited the thermal aggregation of the enzyme at 55°C, whereas at neomycin concentrations greater than 5.2 mM, the modification caused an increase in non-specific

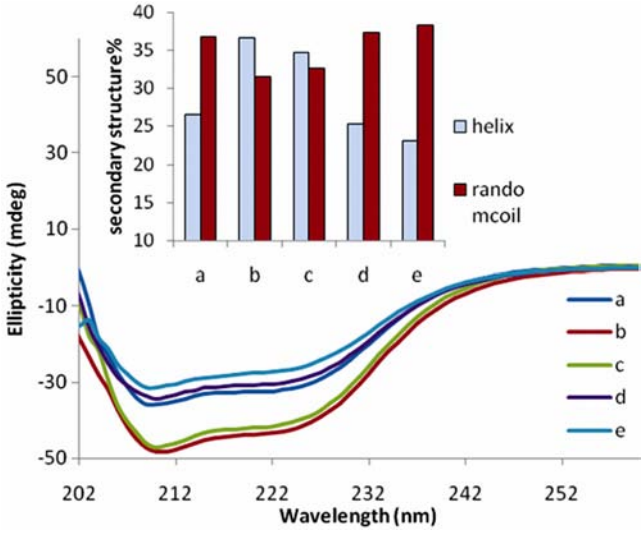

Fig. 5—Far-UV circular dichroism spectra of native CAT (a) and modified CAT with neomycin concentrations of 2.6 mM (b), 5.2 mM (c), 52.0 mM (d), 156.0 mM (e) [The inset shows percentage of the α-helix and random coil secondary structure against neomycin concentration. Experimental conditions: spectra were taken of proteins dissolved at a concentration of 0.2 mg/mL in phosphate buffer (50 mM, pH 7.0)]

thermal aggregation of the enzyme. In order to understand the concentration-dependent effect of neomycin on the thermal aggregation of enzyme, it was necessary to describe the role of factors that influence protein aggregate formation. Among these factors, the most relevant are a high hydrophobicity and a high propensity to convert α-helical to β-sheet structure[30-33].

It seemed that the alterations in hydrophobicity along with changes in structural properties of the modified enzyme at a secondary folding level in a concentration-dependent manner of neomycin were dominant forces in thermal aggregation of the protein. Based on the tryptophan and ANS fluorescence spectra (Figs. 4A and B) at concentrations of neomycin up to 5.2 mM, the chemical modification of carboxyl residues causes the conformational change of the protein at the tertiary level, so that it hides hydrophobic residues and reduces the protein-protein interaction due to loss in hydrophobicity and consequently inhibits thermal aggregation of the protein. Furthermore, as shown in Fig. 5, modification of CAT with neomycin up to 5.2 mM induced an increase in α-helical content of the protein, which could considerably prevent the thermal aggregation. It should be noted that at neomycin concentrations above 5.2 mM, the thermal aggregation increased with decrease in α-helical content of the protein.

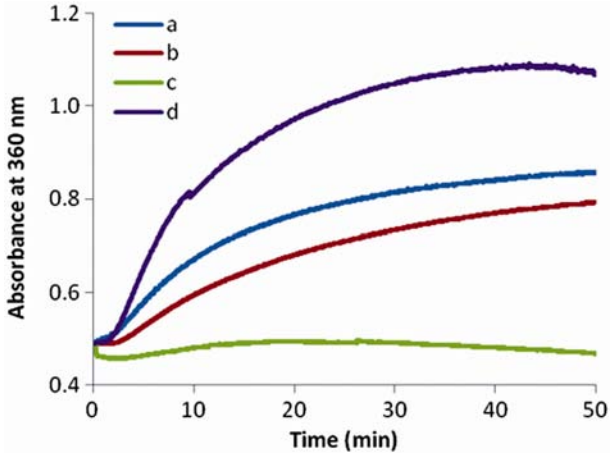

Fig. 6—Aggregation assay at 360 nm of native and modified forms of CAT as a function of time [(a) Native CAT; (b) CAT + neomycin (2.6 mM); (c) CAT + neomycin (5.2 mM); (d) CAT + neomycin (156.0 mM). Experimental conditions: absorbance measurements were carried out with CAT (0.2 mg/mL) in phosphate buffer (50 mM, pH 7.0) at 55°C for 50 min]

Conclusion

The findings of present study suggested that the covalent attachment of neomycin might occur via carboxyl residues of CAT. Our data clearly demonstrated that modification of CAT with different concentrations of neomycin in the range of 0.0-156.0 mM had two different behaviors. CAT modified with neomycin in concentration range 0.0-5.2 mM showed a conformational state, exhibiting little enhancement in CAT activity, higher α-helix content, reduced surface hydrophobicity and better protection against thermal aggregation, while CAT modified with neomycin above 5.2 mM had a conformational state, showing a decrease in the enzyme activity and an increase in the thermal aggregation. This modification indicated that aspartate and glutamate residues had significant role in the structure, function and folding of CAT.

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