Regular paper

Effects of pH on the activity and structure of choline oxidase from *Alcaligenes* species

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A reversible effect of pH on the ionization of amino-acid residues at the active center of choline oxidase was observed near the optimum pH (8). Inactivation of choline oxidase took place in the pH ranges 3–6 and 9–11, in which irreversible changes in the structure occur leading to the enzyme inactivation. The first order rate constants of the enzyme's inactivation at various pH values were estimated for the irreversible changes. The Arrhenius analysis revealed no significant changes in the activation enthalpy, while an increase in the activation entropy reflected an increase in the conformational freedom.

INTRODUCTION

Normally, enzymes are only active over a limited range of pH and in most cases a definite optimum pH is observed. The fall of activity on either side of the optimum may be related to the effect of pH on the stability of the enzyme, which may be irreversibly destroyed on one or both sides of the optimum pH (Dixon & Webb, 1979). The occurrence of irreversible destruction of the enzyme activity can be tested by exposing the enzyme to a range of pH values and then testing the activity after readjusting the pH to some standard value (Dixon & Webb, 1979).

To describe completely the effects of different pH values on the enzyme catalysis is a time-consuming task. Many of the amino-acid side chains in an enzyme are ionizable, but in environments with polarities different from that of the free solution, the pK_a's will probably be significantly altered (Dixon & Webb, 1979). However, experimentally, it is a simple matter to determine values of steady-state kinetic parameters of an enzyme at various pH conditions. The possible effects of pH are due to changes of the ionization state of groups involved in catalysis, groups involved in binding of substrate, groups involved in binding at site(s) other than the active site (*allosteric effector sites*) and groups on the substrates (Kuchel & Ralston, 1988). Michaelis and Davidsohn suggested that the catalytic activity is usually confined to a relatively small range of pH, therefore it seems likely that only one of the many ionic forms of the enzyme (or rather of the active center) is catalytically active (Michaelis & Davidsohn, 1911). There is some evidence that the ionization of the protein groups that are remote from the active center has little or no effect, while the ionic state of groups in or close to the active centre has a very large effect (Kuchel & Ralston, 1988).

Bernheim first demonstrated the presence of choline oxidase in animal tissues (Bernheim & Bernheim, 1933), afterward a great deal of work has been carried out to study this enzyme in details (Williams & Litwack, 1952). Choline oxidase was first isolated from rat liver in 1938 (Mann *et al.*, 1938). At that time, it was publicized that the choline oxidase system was composed of choline dehydroge-

nase, cytochrome *c*, and cytochrome oxidase (Mann *et al.*, 1938). In 1977, Ikuta reported the purification of the enzyme from the soil bacterium *Arthrobacter globiformis* (Ikuta *et al.*, 1977). In addition, the enzyme was purified from other microorganisms, such as *Cylindrocarpon didymium* (Yamada *et al.*, 1979) and *Alcaligenes* species (Ohta-Fukuyama *et al.*, 1980; Ohta *et al.*, 1983). The amino-acid sequence of choline oxidase from *A. globiformis* has been reported, but such information about the choline oxidase from *Alcaligenes* species is not available yet.

Choline oxidase (ChOx, EC 1.1.3.17), a FAD-containing enzyme, catalyzes the oxidation of choline to glycine betaine (*N,N,N*-trimethylglycine) with betaine aldehyde as an intermediate and molecular oxygen as primary electron acceptor (Scheme 1) (Gadda, 2003). The molecular mass of the enzyme was estimated to be 66 kDa for a monomer (Ohta *et al.*, 1983). The prosthetic group of the enzyme from *Alcaligenes* species was identified as 8α-[N(3)-histidyl]-FAD and the partial sequence of amino acids in the flavin peptide reported as Asp-Asn-Pro-Asn-His-(FAD)-Ser-Arg (Ohta-Fukuyama *et al.*, 1980).

$(CH_3)_3N^+CH_2CH_2OH \xrightarrow[O_2 \quad H_2O_2]{} (CH_3)_3N^+CH_2CHO \xrightarrow[O_2,H_2O \quad H_2O_2]{} (CH_3)_3N^+CH_2COOH$

Choline   Betaine aldehyde   Betaine
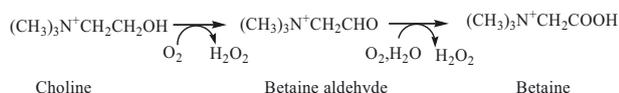
Scheme 1. Reaction catalyzed by choline oxidase.

The investigation on ChOx is of interest to scientists for a number of reasons. The mechanism of the carbon–hydrogen bond cleavage in choline by ChOx is important because of the high energetic barrier associated with this process (Gadda, 2003). This reaction also is of considerable importance for medical and biotechnological reasons, since the accumulation of glycine betaine (GB) has been observed in a number of human pathogenic bacteria and in the cytoplasm of many plants in response to hyperosmotic and temperature stresses, preventing dehydration and cell death. GB also protects the transcriptional and translational machinery by decreasing the melting temperature of double-stranded DNA. This seems to suggest that GB behaves like chaperonin (Graham & Wilkinson, 1992; Bae *et al.*, 1993; Kaenjak *et al.*, 1993; Culham *et al.*, 1994; Deshnium *et al.*, 1995; Deshnium *et al.*, 1997; Alia *et al.*, 1998; Kempf & Bremer, 1998; Peddie *et al.*, 1998; Sakamoto *et al.*, 1998, 2000; Holmstrom *et al.*, 2000). In addition, the development of biosensors for detection of choline, choline derivatives and organophosphorous compounds in biological (Tavakoli *et al.*, 2005) and environmental samples (including air, soil and water) (Nunes & Barcelo, 1998; Guerrieri *et al.*, 2002) render this enzyme of clinical and industrial interest.

Despite the large amount of work, understanding of the detailed effect of different pH values on the activity of choline oxidase is still lacking. This inspired us to study the effect of various pH conditions on the activity of choline oxidase from *Alcaligenes* species. At first, we tested the activity of choline oxidase at different pH values. Since studying the effects of temperature on the enzyme may provide a deeper insight into the physical principles involved in its molecular organization (Sarraf *et al.*, 2004), these measurements were performed at 27 and 37°C. Subsequently, we estimated the first order rate constants of the enzyme's inactivation at various pH conditions.

Afterward we investigated the effects of pH on the structure of choline oxidase. We employed Nile Red as a neutral fluorescence probe to monitor pH-induced changes in the surface hydrophobicity of choline oxidase. These changes can, in principle, produce changes in the position or orientation of the tryptophan residues, altering their exposure to solvent and leading to an alternation in the quantum yield (Ajloo *et al.*, 2007). Subsequently, to study the structural properties of the protein further (Gozdek *et al.*, 2008) at various pH ranges, we carried out far-UV CD (circular dichroism) experiments. Gel electrophoresis of choline oxidase at different pH values was also performed.

It is important to mention that this is the first attempt to study the changes of choline oxidase (from *Alcaligenes* species) activity induced by low and high pH at temperatures of 27 and 37°C. The information obtained in this study will help to understand the effects of pH on the activity and conformation of choline oxidase from *Alcaligenes* species.

MATERIALS AND METHODS

Materials. Choline oxidase (EC 1.1.3.17 from *Alcaligenes* species), choline chloride, horseradish peroxidase (HRP) and Nile Red (9-diethylamino-5*H*-benzo phenoxazine-5-one) were supplied by Sigma (St. Louis, MO, USA). Tris and 4-aminoantipyrene were from BDH Chemicals Ltd. and Aldrich (St. Louis, MO, USA), respectively. Potassium phosphate (K_2HPO_4 and KH_2PO_4) was from Merck (Darmstat, Germany).

Enzyme assay. The initial velocity of the choline oxidase-catalyzed reaction was determined using the procedure described by Sigma-Aldrich in details (Okabe *et al.*, 1977; Keesey, 1987) in a Shimadzu model UV-3100 spectrophotometer, equipped with a temperature control system. A stock solution of ChOx (96.8 μM) was prepared in 10 mM Tris/HCl buffer (pH 8.0) containing 134 mM KCl and 2 mM

EDTA. ChOx was assayed in a mixture containing 1 ml of 100 mM Tris/HCl (pH 8.0), 5 units/mg HRP, 50 mM 4-aminoantipyrine and 150 mM choline chloride as a substrate at 37°C. The reaction was started by the addition of 20 μl of ChOx in a temperature-controlled cuvette compartment. The enzyme reaction was monitored spectrophotometrically at 500 nm by the production of quinonimine dye using an absorption coefficient, $\varepsilon = 12\,000$ M^{-1} cm^{-1} (Okabe *et al.*, 1977; Keesey, 1987). The initial velocity of the reaction was calculated and one unit of enzymatic initial velocity is given as μmol/min.

Choline oxidase (151.14 μM) was dissolved in 100 mM Tris/HCl in the pH range 3–11 and kept at 4°C. The enzyme kept at a given pH for between one and seven days. Every day 18.89 μM of the enzyme at a given pH was readjusted to pH 8.0, and its activity was measured at 27 or 37°C.

Fluorescence measurements. Fluorescence emission spectra were measured in a Hitachi model MPF-4 fluorescence spectrophotometer thermostated with a Protherms bath model NTB-211. The excitation and emission wavelengths were 530 and 550 nm for Nile Red, and 285 and 300 nm for Trp fluorescence. In all cases, 5 and 10 nm excitation and emission slits were used. At high concentrations of the probe, fluorescence was corrected for inner-filter effect.

To study the effect of pH, samples including 30 μM Nile Red and 2 μM choline oxidase in 100 mM Tris/HCl in the pH range 3–11 were prepared. The Nile Red spectra were corrected for the probe's own response to pH in the absent of protein, because Nile Red fluorescence is pH-sensitive. The fluorescence spectrum for each sample was recorded after 30 min incubation at 27 or 37°C.

Circular dichroism (CD) measurements. The secondary structure estimation of choline oxidase was studied using an Aviv circular dichroism spectrometer Model 215. The CD spectrum of choline oxidase at different pH values was recorded from 200 to 250 nm using a quartz cell, 0.1 cm pathlength, with a resolution of 0.2 nm, scan speed of 20 nm min^{-1}, time constant of 2.0 s, 10 nm band width and sensitivity of 20 m. Concentration of choline oxidase in 100 mM Tris/HCl buffer, at different pH, at 27 and 37°C was 41.4 μM. All spectra were corrected by subtracting the proper baseline.

Gel electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was carried out to evaluate the protein at each pH, and SDS/PAGE was used to assess the difference between non-denaturing and denaturing conditions. Assay was performed according to the standard protocol described by Schägger and von Jagow (1987) using 10% acrylamide gel. Protein bands were stained with Coomassie Blue.

RESULTS AND DISCUSSION

The pH-dependent activity changes of choline oxidase

The effect of pH on the activity itself can be determined by carrying out a series of velocity measurements at different pH values using a sufficiently high substrate concentration, which eliminates any effects on the Michaelis constant (K_m). Under these conditions, all enzyme is in the form of the ES complex, and since the velocity of the reaction (*V*) is simply the rate of breakdown of the complex into free enzyme and the product, the effect of pH on the velocity will be determined only by the state of ionization of the complex (Dixon & Webb, 1979). Changes in the state of ionization of the free enzyme
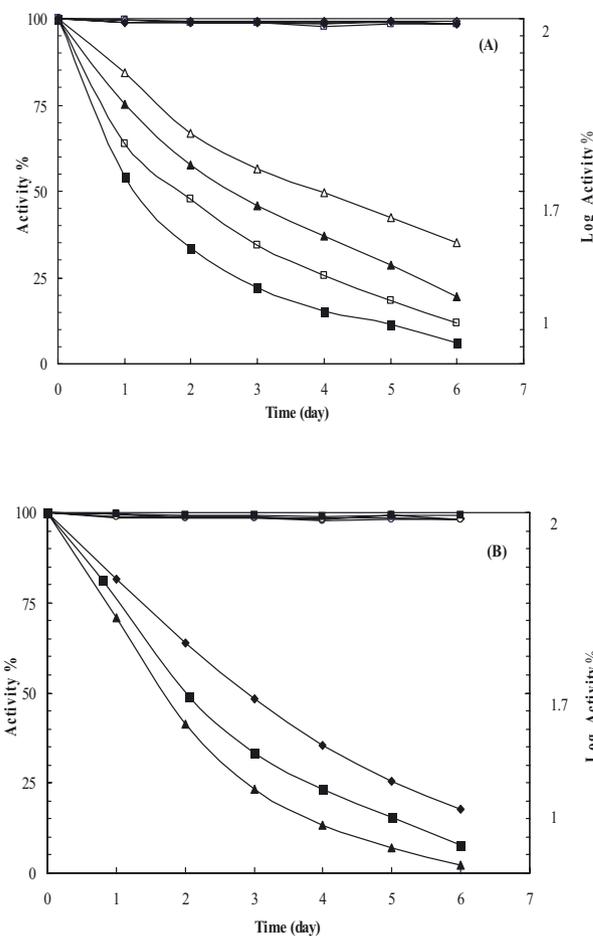
Figure 1. Effects of pH on the activity of choline oxidase at 27°C.
Panel A: Activity at acidic pH, (■) pH 3, (□) pH 4, (▲) pH 5, (△) pH 6 and the activity near neutral pH, (◆) pH 7, (✕) pH 7.5, (✛) pH 8, (●) pH 8.5. Panel B: activity near neutral pH, (◆) pH 7, (✕) pH 7.5, (✛) pH 8, (●) pH 8.5 and the activity at alkaline pH, (◆) pH 9, (■) pH 10, (▲) pH 11 (at pH 7, 7.5, 8 and 8.5 all lines are almost horizontal and they strongly overlap). Activity was measured as described in Materials and Methods.

or substrate will not affect V; they will affect K_m, but the effects on K_m are eliminated by the use of high substrate concentrations (Dixon & Webb, 1979). The protonation of a basic group on an enzyme is simply a special case of the binding of a modifier at the specific site. However, there are several differences between protons and other modifiers that make it worth examining protons separately. First, protons affect virtually all enzymes, so that the proton concentration can be measured and controlled over a range that is enormously greater than that available for any other modifier, and therefore one can expect to observe very versatile effects on enzyme kinetics. In addition, protons normally bind to many sites on an enzyme, so that it is often appropriate to consider binding at one site only (Dixon & Webb, 1979).

The effects of various pH conditions were tested by exposing choline oxidase in the pH range
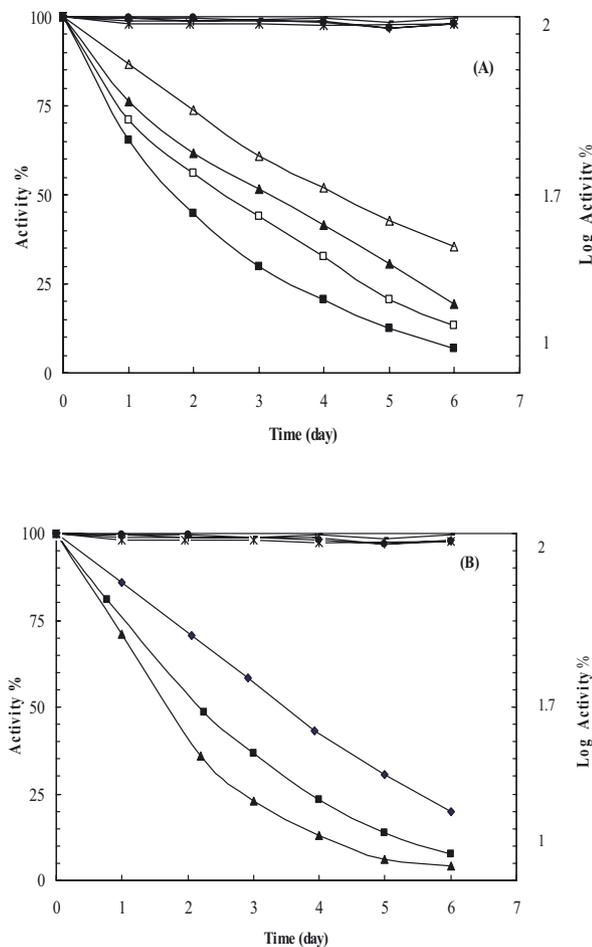
3–11 and then testing the activity after readjusting the pH to the standard value (pH 8.0). To illustrate the time dependence of inactivation at various pH values, the activity percentage and the log activity percentage were measured during one week of enzyme pre-incubation at 27 (Fig. 1) and 37°C (Fig. 2) using high substrate concentrations to eliminate the effect of pH on K_m.

The time-course of the changes of choline oxidase activity at various pH values as shown in Figs. 1 and 2 revealed a more rapid inactivation at alkaline than at acidic pH. It is interesting to point out that at four pH values (7.0, 7.5, 8.0 and 8.5) no significant inactivation was observed. Thus, the inactivation of choline oxidase took place in the pH ranges 3–6 and 9–11.

In order to study the occurrence of irreversible changes of choline oxidase, the enzyme was exposed to a pH range 3–11 and analyzed by native PAGE. The analysis was performed after 30 min incubation (Fig. 3). The enzyme was also analyzed by SDS/PAGE (Fig. 4). Recently the crystal structure of choline oxidase from *Arthrobacter globiformis* was determined and indicated that the enzyme has a dimeric structure under non-denaturing conditions. The molecular mass of choline oxidase from *Alcaligenes* species was also estimated to be 66 kDa for a monomer (Quaye *et al.*, 2008; Ohta *et al.*, 1983). Accordingly, native gel electrophoresis shown in Fig. 3 demonstrates that the enzyme displays no transition from a dimer to monomer at different pH values (the molecular mass of the enzyme is 120 kDa),

Figure 2. Effects of pH on the activity of choline oxidase at 37°C.
Panel A: Activity at acidic pH, (■) pH 3, (□) pH 4, (▲) pH 5, (△) pH 6 and the activity near neutral pH, (◆) pH 7, (✗) pH 7.5, (✚) pH 8, (●) pH 8.5. Panel B: activity near neutral pH, (◆) pH 7, (✗) pH 7.5, (✚) pH 8, (●) pH 8.5 and the activity at alkaline pH, (◆) pH 9, (■) pH 10, (▲) pH 11 (at pH 7, 7.5, 8 and 8.5 all lines are almost horizontal and they strongly overlap). Activity was measured as described in Materials and Methods.

Figure 3. Native PAGE of choline oxidase pre-incubated at various pH values.
Choline oxidase was pre-incubated at different pH conditions for 30 min and analysed by native PAGE according to the standard protocol described by Schägger and von Jagow (1987). M: molecular mass marker (Tyrosinase).
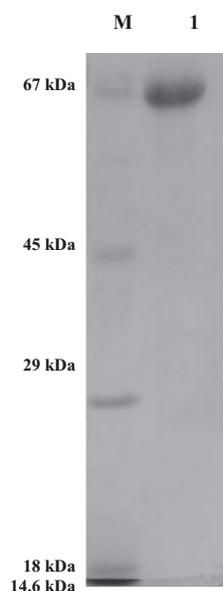
Figure 4. SDS/PAGE profile of choline oxidase.
The assay was performed according to the standard protocol described by Schägger and von Jagow (1987) using 10% acrylamide gel. Protein bands were stained with Coomassie Blue. Lane 1 represents choline oxidase at pH 8.0. M: molecular mass marker.

whereas SDS/PAGE analysis (Fig. 4) reveals that the enzyme forms monomers in denaturing condition, thus the enzyme maintains its dimeric structure at various pH conditions.

It was proposed that the hydride transfer reaction catalyzed by choline oxidase under irreversible regime, i.e., at saturating oxygen, occurs quantum mechanically within a highly reorganized active site (Fan *et al.*, 2007). Moreover, the pH dependence of the deuterium isotope effects on the maximal velocity (V_{max}) value and V_{max}/K_m values for choline clearly indicate that an amino-acid group with an apparent pK_a value about 7.5 must be unprotonated for catalysis (Gadda, 2003). This catalytic base is essential for enzyme-catalyzed oxidation of choline to glycine betaine, furthermore, it is required for the formation of a highly reactive choline-alkoxide species (Fan *et al.*, 2007). Results showed that when the pH is at least one unit below the pK_a value of this active site base, cleavage of the carbon–hydrogen bond becomes fully rate limiting for catalysis (Gadda, 2003). The pH effects on the velocity of enzyme reactions are frequently discussed in terms of ionizing groups. Consequently, one of the possible effects of pH is to change the ionization state of groups involved in catalysis, groups involved in binding of substrate and groups on the substrates. No ionizable groups are present on the substrate choline with the exception of the hydroxyl group. Hence, we suggest that the possible reasons for inactivation of the enzyme at low pH values are changes in the ionization state of the catalytic base involved in catalysis, disturbance
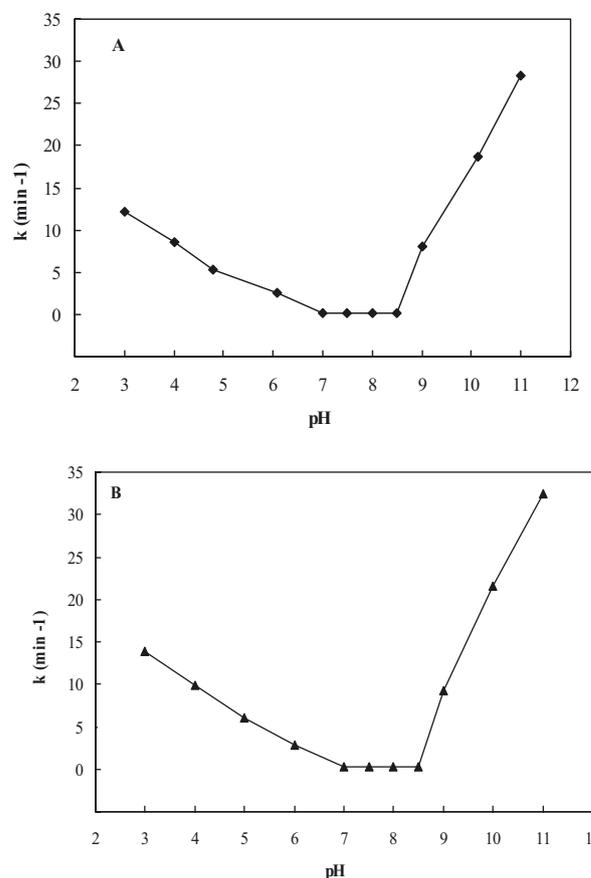
Figure 5. First order rate constants of choline oxidase at various pH values at 27 and 37°C.
The first order rate constants at various pH values were estimated by measuring the slope of each graphs of Fig. 1 and 2 and multiplying them by (–2.3). **(A)** values at 27°C, **(B)** values at 37°C.

of the hydride transfer reaction catalyzed by choline oxidase and unsuitable configuration for tunneling of the hydride transfer reaction.

The first order rate constants at various pH values were estimated at 27 and 37°C by measuring the slope of each graph of Figs. 1 and 2, respectively, and multiplying them by (–2.3) (Figs. 5A and B). Subsequently, the thermodynamic parameters dependent on pH and temperature were analyzed in order to further characterize the effect of these environmental changes. The acting forces between a small molecule and a macromolecule mainly include hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interaction forces (Khan *et al.*, 2008). The thermodynamic parameters, enthalpy change ($\Delta H°$) and entropy change ($\Delta S°$), are the main components that determine the effect of high and low pH. By using the Arrhenius equation (Eqn. 1), the activation enthalpies of the reaction catalyzed by choline oxidase were calculated at each pH. Subsequently, $\Delta S°$ (Y-intercept) for each pH was estimated using Eqn. 2:

$$E_a = \frac{RT_1T_2}{T_2 - T_1} \ln \frac{k_2}{k_1} \tag{1}$$

$$\ln k = \frac{-\Delta H^\circ}{R} \frac{1}{T} + \frac{\Delta S^\circ}{R} \tag{2}$$

where k represents the first order rate constant of protein inactivation at absolute temperature T, and R is the universal gas constant. The thermodynamic parameters are given in Table 1.

Accordingly, the rates of oxidation of choline by choline oxidase increase with a rise of 10°C in temperature, but no significant difference in ΔH° at each pH was observed, whereas ΔS° showed significant changes occurring at high pH values due to changes in the hydrophobic interaction and an increase in the conformational freedom.

Fluorescence measurements

Several studies have identified surface hydrophobic sites on proteins by observing the fluorescence enhancement of polarity-sensitive dyes like ANS on addition to proteins. Since ANS (8-anilino-1-naphthalenesulfonate) is a negatively charged probe, electrostatic interactions may play a role in its binding to the protein. Furthermore, it has been demonstrated that the probe itself can induce conformation changes in proteins (Ali *et al.*, 1999). In order to exclude these possibilities, we used another polarity-sensitive probe, neutral Nile Red. Many reports in the literature confirm that the probes' quantum yields depend strongly on the polarity of their environment: their fluorescence increases upon binding to hydrophobic sites on the protein (Cardamone & Puri, 1992; Kotik & Zuber, 1993). Consequently, intrinsic fluorescence emission spectra of choline oxidase in the presence of Nile Red at different pH values and fluorescence emission spectra of Nile Red after exposing choline oxidase to pH in the range between 3 and 11 at 27 and 37°C were studied. The data were corrected for the probe's own response to pH in the absence of protein. Figure 6 shows plots of the maximum emission intensity at

Table 1. Thermodynamic parameters of activity of choline oxidase at various pH values

pH	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
3.0	10.89	96.56
4.0	10.89	95.73
5.0	10.89	86.16
6.0	10.89	76.59
7.0	10.89	42.12
7.5	10.89	42.12
8.0	10.89	42.12
8.5	10.89	42.12
9.0	10.89	99.82
10.0	10.89	105.31
11.0	10.89	114.88
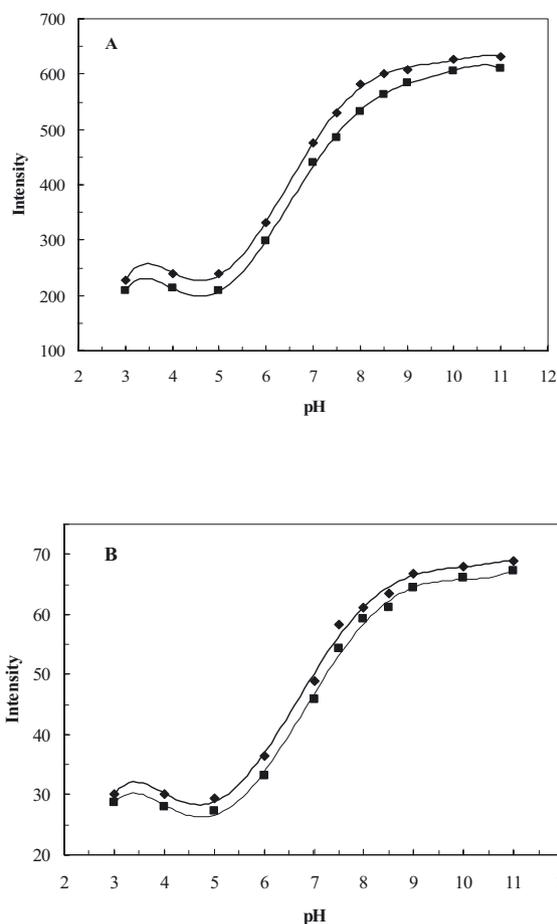
Figure 6. Intrinsic and extrinsic fluorescence of choline oxidase–Nile Red complex as a function of pH.
(A) pH dependence of the fluorescence intensity of Nile Red in the presence of choline oxidase at 27 (◆) and 37°C (■). Concentration of the dye was 30 μM (Nile Red) and that of the enzyme was 2 μM. **(B)** pH dependence of Trp fluorescence in the presence of Nile Red at 27 (◆) and 37°C (■).

341 and 610 nm at 27 and 37°C corresponding to the intrinsic and extrinsic fluorescence emission of choline oxidase in the presence of Nile Red.

As shown in Fig. 6, in the pH range between 3 and 5 the quantum yield remained at low levels but at higher alkaline pH values the quantum yield of the probe tripled. In other words, these figures demonstrate an increase in the fluorescence by increasing pH values, which approaches a maximum at pH 11.

The observed increase in the fluorescence intensity is due to increased binding of Nile Red, which is a result of exposure of buried hydrophobic amino-acid residues on the protein surface. This result is consistent with an increase in the activation entropy at high pH values (Table 1). According to Fig. 6, it is interesting to point out that at pH 8 the protein has more accessible hydrophobic patches than at acidic pH.

Circular dichroism measurements

From a fundamental standpoint, changes in pH can bring about changes in enzyme conformation, which in turn can affect its interaction with the substrate and its activity. In fact, protein unfolding and denaturation at low or high pH is quite a general phenomenon. CD has proved to be an ideal technique to monitor conformational changes in proteins, which can occur as a result of changes in experimental parameters such as pH, temperature, binding of ligands and so on (Kelly & Price, 2000). To investigate the effects of low and high pH values that cause choline oxidase inactivity, far-UV CD spectra (190–260 nm) were used for determination of the secondary structure of the protein. The peptide bond is the principal absorbing group and studies in this spectral region can give information on the secondary structure (Sreerama & Woody, 2004; Saboury *et al.*, 2004). Figure 7 shows far-UV CD spectra of the enzyme incubated at different pH values.

Table 2. Content of the secondary structure of choline oxidase at different pH values at 27°C

Random coil (%)	β-Sheet (%)	α-Helix (%)	pH
8.7	9.2	82.1	3
11.2	11	77.8	4
19.4	13.2	67.4	6
13	9	78	8
33	35	32	9
46.4	44	9.6	10

The spectra of the enzyme at pH 8 at both 27 and 37°C show significant negative bandwidth double minima at 208 and 222 nm, which are the characteristics of α-helix (Fig. 7d). Tables 2 and 3 indicate the content of the secondary structures of choline oxidase under various pH values at 27 and 37°C. Interestingly, high alkaline pH (pH 9 and 10) affected the secondary structure of choline oxidase and a transition from α-helix to β-structure was visible (Fig 7A, B and Tables 2 and 3). It should be pointed out that at very acidic pH values (pH 3 and 4) the content of α-helical structure significantly increased (Fig 7e, f and Tables 2 and 3). By comparing the content of α-helical structure at very acidic pH and pH 8, we can conclude that the enzyme at pH 8 is more flexible than at very acidic pH. Figure 7 confirms that various pH values caused changes in the secondary structure of choline oxidase. It also indicates that with a rise of 10°C in temperature, the overall shape of the spectra do not change significantly.

It is important to point out that transition from α-helix to β-structure appears to be physiologically important. The conformational switch from the α-helix to β-sheet may lead to the formation of amyloid structures (Bokvist *et al.*, 2004). In addition, the α-helix to β-sheet conformational transition(s) has been shown in Alzheimer's AB peptide (Divsalar *et al.*, 2006). On the other hand, at lower pH values the content of α-helix structure was increased (Fig. 7 and Tables 2 and 3) and consequently prevented the access of the substrate (choline chloride) to the enzyme's active site. Taken together, the conclusion is reached that different pH values can bring about changes in enzyme structure which in turn can af-

Figure 7. CD spectra of choline oxidase pre-incubated at various pH values.
Far-UV circular dichroism spectra were determined for 41.4 µM choline oxidase after incubation at pH 10 (a), 9 (b), 6 (c), 8 (d), 4 (e) and 3 (f) at 27 °C **(A)** or 37°C **(B)**.

Table 3. Content of the secondary structure of choline oxidase at different pH values at 37°C

Random coil (%)	β-Sheet (%)	α-Helix (%)	pH
9.1	9.4	81.5	3
13.4	9.3	77.3	4
19.1	13.1	67.8	6
15	8	77	8
35	32	33	9
45.8	45	9.2	10

fect choline oxidase interaction with the substrate (choline chloride), and its activity.

CONCLUSION

In the present study, the inactivation of choline oxidase from *Alcaligenes* species at various pH values was studied. No significant inactivation was seen when the enzyme was incubated at pH between 7 and 8. However, inactivation of choline oxidase took place in the pH ranges 3–6 and 9–11, which was related to irreversible changes in the structure of the enzyme. No significant change in the activation enthalpies of the reaction catalyzed by choline oxidase occurred on raising the temperature by 10°C at various pH(s), and the entropy demonstrated significant change suggesting alteration in hydrophobic interactions and increase in conformational freedom at higher pH values.

Native PAGE at various pH values showed that the enzyme was in its dimeric structure. The interaction of an extrinsic probe (Nile Red) with choline oxidase in solution was investigated using fluorescence techniques. Nile Red fluorescence is very environmentally sensitive and the presence of domains of differing polarity within the enzyme was ascertained by the decomposition of the Nile Red emission spectrum. Thus we discovered that at both 27 and 37°C with increasing pH, Nile Red affinity toward the hydrophobic patch of the enzyme increased. This result is consistent with the data from activation entropies estimation and confirms that the enzyme at high alkaline pH values has more accessible hydrophobic patches relative to acidic pH.

Far-UV CD studies of choline oxidase at different pH values showed a substantial effect of pH on the secondary structure of the protein. The native protein has α-helical structure while at higher alkaline pH a transition from α-helix to β-structure appeared. On the other hand, at lower pH values the content of α-helix structure was increased and consequently prevented the access of the substrate to the enzyme active site.

Combining the results from activity measurements, extrinsic probe (Nile Red) fluorescence emission and CD measurements, we conclude that the activity and structure of choline oxidase is modulated by pH. In other words, low and high pH induced significant conformational changes in choline oxidase causing irreversible inactivation of this. These results provide useful information to design better biosensors for detection of choline, choline derivatives and organophosphorous compounds in biological and environmental samples and for developing curative agents that can specifically inhibit the formation of glycine betaine and render pathogens more susceptible to conventional treatment.

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