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Structural and functional changes of bovine carbonic anhydrase as a consequence of temperature✪

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The temperature dependence of the activity and structure of the enzyme carbonic anhydrase was studied. The Arrhenius plot shows a jump which is seen usually in proteins with more than one subunit or with one subunit but more than one domain. Since carbonic anhydrase has only one subunit with one domain, the fine conformational changes of the protein motifs could only be detected through circular dichroism polarimetry. It seems that the jump in Arrhenius plot is a result of some slight structural changes in the secondary and tertiary structures of the enzyme.

The zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) has been intensely investigated since its discovery (Meldrum & Roughton, 1933; Liljas *et al.*, 1994). This ubiquitous enzyme is present in archaeo and eubacteria, green plants and animals (Supuran & Scozzafava, 2000; Supuran *et al.*, 2003). CA catalyses a variety of reactions, including the reversible hydration of CO_2 to bicarbonate, as a physiological reaction, and some other less investigated processes (Pocker & Stone, 1967; Sly & Peiyi, 1995; Supuran *et al.*, 1997) such as hydrolysis of aromatic and aliphatic esters, or the hydration of cyanate to urea, etc.

The primary structure of CA in bovine, sheep and other mammals is very similar to each other and to that of the human enzyme (Lindskog *et al.*, 1971). The amino-acid sequences of human and bovine carbonic anhydrase are almost 87 percent identical.

The crystal structures of many isoenzymes of CA have been reported (Lindskog, 1997; Lehtonen *et al.*, 2004). The overall structures

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Abbreviations: bCA, bovine carbonic anhydrase.

of these isoenzyme forms are very similar. With the exception of the amino terminal region (about 24 amino acids) which has few contacts with the rest of the protein (Aronsson *et al.*, 1995) the enzyme might be considered as a one-domain protein (Hakansson *et al.*, 1992; Aronsson *et al.*, 1995; Lindskog, 1997) with no disulfide bonds. CA has some helical structure and a dominating β-sheet that extends throughout the entire molecule (Hakansson *et al.*, 1992; Freskgard *et al.*, 1994; Aronsson *et al.*, 1995). A zinc ion is situated at the bottom of a 1.5 nm deep active site cleft and is coordinated to three histidines (His 94, His 96 and His 119) and a water molecule/hydroxide ion (Bertini *et al.*, 1992; Supuran *et al.*, 2003).

As this enzyme is involved in crucial physiologic or pathologic processes (Supuran *et al.*, 2001; Supuran & Scozzafava, 2001) the study of its kinetics and thermodynamic properties is important. Besides, the study of the effects of temperature on the enzyme, including some structure-related properties, may provide a deeper insight into the physical principles involved in the molecular organization of proteins (Lavecchia & Zugaro, 1991). So in this study, the effect of temperature on the activity and relevant structural changes of this enzyme are studied in some detail.

MATERIALS AND METHODS

Erythrocyte bovine carbonic anhydrase (bCA) and *p*-nitrophenylacetate were obtained from Sigma. The buffer used in the assays was 50 mM Tris, pH 7.5, which was obtained from Merck.

Enzyme activity was assayed using a spectrophotometer with jacketed cell holders by following the increase in absorbance at 400 nm due to the production of *p*-nitrophenol (Pocker & Stone, 1967). Its temperature was regulated by an external thermostated water circulator within ± 0.05°C. Enzyme solution was pre-incubated for 5 min at the assay temperature and then initial rates of *p*-nitrophenylacetate hydrolysis catalyzed by bCA were determined at various temperatures. Enzyme and substrate concentrations were 0.3 μM and 6 mM, respectively. Nonenzymatic hydrolysis rate was always subtracted from the observed rate (Sarraf *et al.*, 2002). Care was taken to use adequate experimental conditions to keep the enzyme reaction linear during the first minute of reaction.

In temperature-scanning spectroscopy, absorbance profiles, which describe the thermal denaturation of bCA, were obtained with a UV/visible spectrophotometer CARY-100-Bio model fitted with a temperature programmer which controls the speed of temperature change in denaturation experiments. The cuvette holder can accommodate two samples: a reference buffer solution and a sample for experimental determination. The sample cell contained 10.3 μM of bCA. The recording chart reads the temperature reference line (from the reference cuvette) and the absorbance change at 280 nm in the sample cuvette.

Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectrophotometer (Japan). The results were expressed as ellipticity [θ(deg cm^2 dmol^{-1})] based on a mean amino-acid residue weight (MRW) of 112 for the enzyme and the molecular mass of 29 kDa (Wong & Hamlin, 1974). The molar ellipticity was determined as $[\theta]_\lambda = (100 \times \text{MRW} \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path in cm. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2 dmol^{-1} (Schippers & Dekkers, 1981), and Jasco standard nonhydroscopic ammonium (+)-10-camphorsulfonate assuming $[\theta]_{290.5} = 7910$ deg cm^2 dmol^{-1} (Takakuwa *et al.*, 1985; Protasevich *et al.*, 1997). The noise in the data was smoothed by using the Jasco J-715 software. This software uses the fast Fou-

rier-transform noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes. Determination of the secondary structure of bCA was done using the software "SSE-338" model designed by Jasco company (Japan), according to the Yang statistical algorithm, which deconvolutes far-UV CD spectrum (Yang *et al.*, 1986; Marthasarathy & Johnson, 1987). CD experiments were carried out two times, with the results showing no significance difference.

Far-UV CD was carried out in the presence of 6.7 μM bCA. In the near-UV region higher concentrations of protein should be used due to the low absorption coefficients of the chromophores. Thus 39.3 μM bCA was applied in near-UV CD experiments. Besides, detailed structural differences from the far-UV CD spectrum of bCA were calculated (Kelly & Price, 1997).

The spectrophotometer is fitted with a temperature programmer, which controls the speed of temperature changes in denaturation experiments. The scan rate was set at 2 K min^{-1}. The denaturation of secondary and tertiary structures was followed by measuring the ellipticity changes at 222 and 272 nm, respectively.

RESULTS AND DISCUSSION

The specific activities of bCA at different temperatures were obtained. Figure 1 shows the Arrhenius plot for bCA. The plot depicts two distinct lines with different slopes. The break in the Arrhenius plot is between 27 and 30°C. The activation energy of bCA is 4.69 kJ mol^{-1} below 27°C and 1.45 kJ mol^{-1} above 30°C, obtained from the slope of the Arrhenius plot.

Nonlinear Arrhenius plots have been reported for many enzymes. This nonlinearity can appear as a curvature (Allen *et al.*, 1990), a break (Massey *et al.*, 1966) or a jump (Moosavi-Nejad *et al.*, 2001). In some cases an

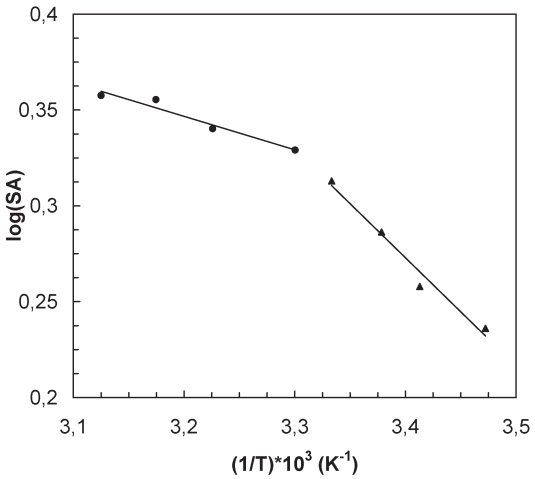

Figure 1. Arrhenius plot, logarithm of specific activity *versus* temperature, for bCA in 50 mM Tris buffer, pH 7.5.

enzyme may show different forms of the Arrhenius plot with different substrates (Allen *et al.*, 1990). There are two main reasons possible for a non-linear Arrhenius plot (Massey *et al.*, 1966; Biosca *et al.*, 1983). First of all, there may be a change in the rate-limiting step of enzymatic reaction. Enzyme reaction pathways consist of some steps, each described by rate constants and characteristic energies of activation. As the temperature is varied, a change in the rate-limiting step can occur. So, the nonlinearity is due to purely kinetic phenomena without any changes in the conformation of the enzyme's active site (Allen *et al.*, 1990). Another reason is a temperature-induced conformational change in a soluble enzyme or phase change in a membrane-associated enzyme, or both. The difference in activation energies (ΔE_a) for such a system is usually high, and the relevant conformational change in the enzyme has been confirmed by following at least one physical property change with temperature using different techniques such as CD polarimetry (Allen *et al.*, 1990), UV/Vis spectrophotometry (Massey *et al.*, 1966), differential scanning calorimetry (Moosavi-Nejad *et al.*, 2001) or turbidity (Biosca *et al.*, 1983).

For the enzyme bCA it is clear from the Arrhenius plot that in the range of 15–27°C,

the activation energy is obviously higher than in the range of 30–47°C, although in both ranges the activity increases with temperature. Observing any structural changes in the enzyme with at least one of the previously mentioned techniques would help in understanding the reasons for the observed duality. However, since bCA is a one-subunit and one-domain protein, it may seem difficult to explain its non-linear activity behavior with temperature. So, one may decide to search for the conformational changes among the motifs of the enzyme by CD polarimetry.

Generally speaking, bCA thermal denaturation curve has a classical sigmoidal shape, indicating a T_m of 64.2°C. If one wants to take a deeper look into the thermal denaturation pattern of bCA, it is possible to study this process *via* far and near-UV CD spectrophotometry. In Fig. 2 the thermal denaturation plot of bCA, *via* UV/Vis and CD spectrophotometry is shown. The overall T_m of the enzyme is 64.2°C obtained *via* UV/Vis. The T_m of secondary and tertiary structures are 68.5 and 62.8°C, respectively, obtained by CD. These data are reasonable because of the more fragile nature of the tertiary structure of the protein comparing to the secondary structure. Although the T_m of the secondary structure (68.5°C) is higher than that of tertiary structure (62.8°C) of the enzyme, a gradual increase in θ value with increasing temperature is observed in the pre-transition step of secondary structure melting (see Fig. 2b), which is not observed in the pre-transition step of tertiary structure melting (see Fig. 2c). Hence, a small change in secondary structure occurs at pre-transition temperatures. Following the CD spectra in far and near-UV region at some temperatures of the pre-transition step, it is possible to catch the differences more thoroughly (Fig. 3). These temperatures are selected with respect to the Arrhenius plot of the enzyme, so that each temperature belongs to one of the specific parts of the plot, either on the lines (25 and 40°C), or outside (52°C).

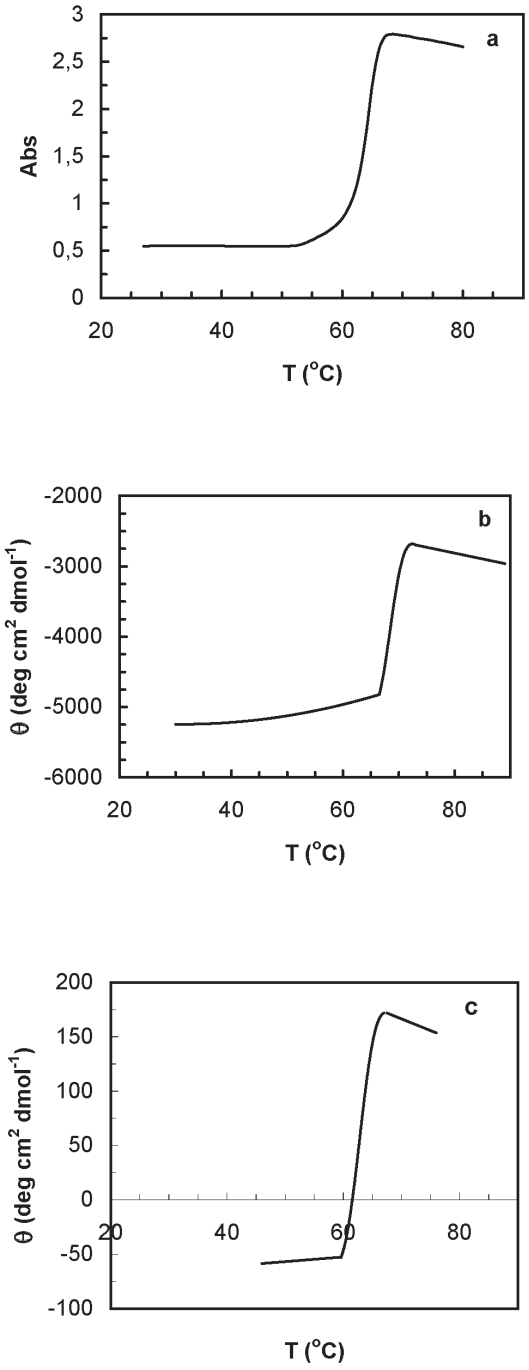

Figure 2. Profiles of thermal denaturation of bCA in 50 mM Tris buffer, pH 7.5, detected by UV/Vis spectrophotometry at 280 nm (a) and circular dichroism at 222 (b) and 272 nm (c).

The near-UV CD spectrum of bCA shows changes of θ in all the specific regions for Phe, Tyr and Trp due to the change in temperature. The change in θ between 40° and 52°C is more obvious than between 25° and 40°C, which means that the tertiary structure of the

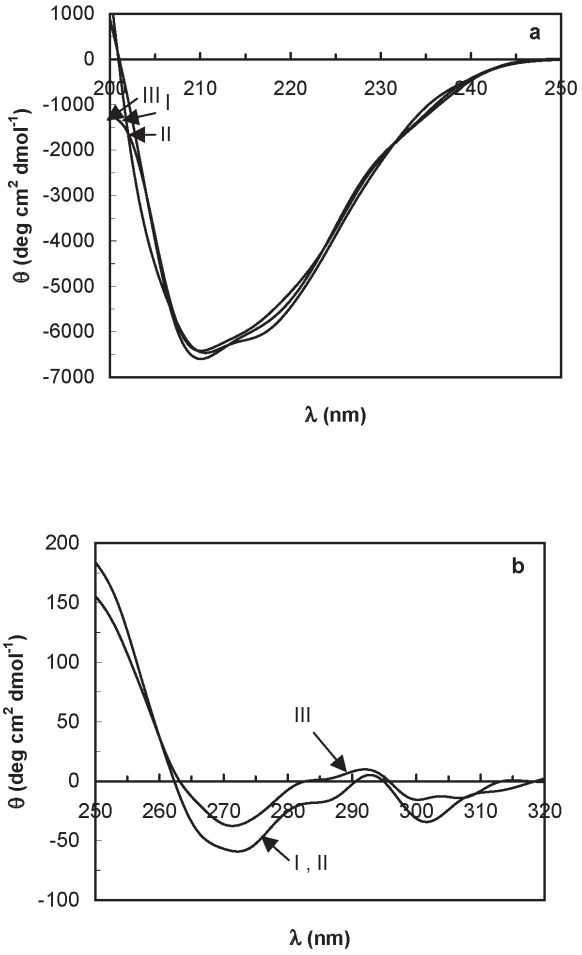

Figure 3. Circular dichroism spectra of bCA in 50 mM Tris buffer, pH 7.5, at different temperatures: (I) 25°C, (II) 40°C and (III) 52°C. (a) Far-UV CD spectrum. (b) Near-UV CD spectrum.

enzyme is maintained from 25°C to 40°C and becomes looser as the temperature is increased to 52°C. So it seems that two distinct lines with different slopes in the Arrhenius plot would not be very much relevant to the tertiary structural change. Detailed structural differences from the far-UV CD spectrum of bCA were calculated and recorded in Table 1 (the results were identical in two CD experiments). It seems that there are a decrease and an increase in the amount of β-structures and random coil, respectively, from 25 to 40°C. But from 40 to 52°C, the amount of helix is slightly decreased and there is an increase in the percentage of β-structures. The first decrease may be due to the lower stability of β-structures comparing to helices. But at higher temperatures the structure of the enzyme becomes looser and there may be two possibilities. First, some of the amino acids may liberate from their relevant hydrogen bond in the protein structure. Thus it may be possible for them to take part in another hydrogen bond which may be considered as a part of a β-structure this time. From another point of view, at temperatures above 40°C, establishment of β-structures may become more favorable. For instance, some fine movements of amino acids due to the increasing of temperature and liberating some hydrogen bonds may place them in some situations, which is more favorable for formation of β-structures.

In conclusion, it can be seen that single-domain enzymes like carbonic anhydrase, due to slight conformational distortions as an effect of temperature, may have a special form of Arrhenius plot, such as one with a "jump", which is usually seen in proteins with more than one domain or subunit. This phenomenon may be important in the cases where it is necessary to select the proper temperature for getting a derived effect.

Table 1. Secondary structure content as a function of temperature in bCA in 50 mM Tris buffer, pH 7.5

T (°C)	Helix (%)	β-structures (%)	Random Coil (%)
25	21.5 ± 0.2	34.6 ± 0.2	43.9 ± 0.2
40	21.7 ± 0.2	32.4 ± 0.2	45.9 ± 0.2
52	19.9 ± 0.2	34.2 ± 0.2	46.9 ± 0.2

REFERENCES

Allen B, Blum M, Cunningham A, Tu GC, Hofman T. (1990) A ligand-induced, temperature dependent conformational change in penicillopepcin. *J Biol Chem.*; **265**: 5060–5.

Aronsson G, Martesson LG, Carlsson U, Jonsson BH. (1995) Folding and stability of the N-terminus of human carbonic anhydrase II. *Biochemistry.*; **34**: 2153–62.

Bertini I, Luchinat C, Pieratelli R, Vila AJ. (1992) Interaction of acetate and formate with Co-carbonic anhydrase. An NMR study. *Eur J Biochem.*; **208**: 607–15.

Biosca JA, Travers F, Barman TE. (1983) A jump in an Arrhenius plot can be consequence of phase transition. *FEBS Lett.*; **153**: 217–20.

Freskgard PO, Martensson LG, Jonasson P, Jonasson BH, Carlsson U. (1994) Assignment of the contribution of the Trp residues to the circular dichroism spectrum of human carbonic anhydrase II. *Biochemistry.*; **33**: 14281–8.

Hakansson K, Carlsson M, Svensson LA, Liljas A. (1992) Structure of native and apo carbonic anhydrase II and structure of some of its anion-ligand complexes. *J Mol Biol.*; **227**: 1192–204.

Kelly SM, Price NC. (1997) The application of circular dichroism to studies of protein folding and unfolding. *Biochim Biophys Acta.*; **1338**: 161–85.

Lavecchia R, Zugaro M. (1991) Thermal denaturation of bovine carbonic anhydrase. *FEBS Lett.*; **292**: 161–4.

Lehtonen J, Shen B, Vihinen M, Casini A, Scozzafava A, Supuran CT, Parkkila A-K, Saarnio J, Kivela AJ, Waheed A, Sly WS, Parkkila S. (2004) Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. *J Biol Chem.*; **279**: 2719–27.

Liljas A, Hakansson K, Jonsson BH, Xue Y. (1994) Inhibition and catalysis of carbonic anhydrase. *Eur J Biochem.*; **219**: 1–10.

Lindskog S. (1997) Structure and mechanism of carbonic anhydrase. *Pharmacol Ther.*; **74**: 1–20.

Lindskog S, Handerson LE, Kannan KK, Liljas A, Nyman PO, Strandberg B. (1971) Carbonic anhydrase. In *The Enzymes,* Boyer PD, ed; **5**: pp 587–665.

Marthasarathy P, Johnson WC Jr. (1987) Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. *Anal Biochem.*; **167**: 76–85.

Massey V, Curti B, Ganther H. (1966) A temperature-dependent conformational change in D-amino acid oxidase and its effect on catalysis. *J Biol Chem.*; **241**: 2347–57.

Meldrum NU, Roughton FJW. (1933) Carbonic anhydrase; its preparation and properties. *J Physiol (London).*; **80**: 113–41.

Moosavi-Nejad SZ, Rezaei-Tavirani M, Padiglia A, Floris G, Moosavi-Movahedi AA. (2001) Amine oxidase from lentil seedling: energetic domains and temperature effect on activity. *J Prot Chem.*; **20**: 405–11.

Pocker Y, Stone JT. (1967) The catalytic versatility of erythrocyte carbonic anhydrase III: Kinetic studies of the enzyme catalyzed hydrolysis of *p*-nitrophenyl acetate. *Biochemistry.*; **6**: 668–78.

Protasevich I, Ranjbar B, Labachov V, Makarov A, Gilli R, Briand C, Lafitte D, Haiech J. (1997) Conformational and thermal denaturation of apocalmodulin: role of electrostatic mutations. *Biochemistry.*; **36**: 2017–24.

Sarraf NS, Saboury AA, Moosavi-Movahedi AA. (2002) Product inhibition study on carbonic anhydrase using spectroscopy and calorimetry. *J Enzyme Inhib Med Chem.*; **17**: 203–6.

Schippers PH, Dekkers HPJM. (1981) Direct determination of absolute circular dichroism data and calibration of commercial instrument. *Anal Chem.*; **53**: 778–88.

Sly WS, Peiyi HY. (1995) Human carbonic anhydrase and carbonic anhydrase deficiencies. *Annu Rev Biochem.*; **64**: 375–401.

Supuran CT, Conroy CW, Maren TH. (1997) Is cyanate a carbonic anhydrase substrate? *Proteins Struct Func Genet.*; **27**: 272–8.

Supuran CT, Scozzafava A. (2000) Carbonic anhydrase inhibitors and their therapeutic potential. *Exp Opin Ther Patent.*; **10**: 575–600.

Supuran CT, Scozzafava A. (2001) Carbonic anhydrase inhibitors. *Curr Med Chem-Imm Endoc Metab Agents*; **1**: 61–97.

Supuran CT, Briganti F, Tilli D, Chegwidden WR, Scozzafava A. (2001) Carbonic anhydrase inhibitors: Sulfonamides as antitumor agents? *Bioorg Med Chem.*; **9**: 703–14.

Supuran CT, Scozzafava A, Casini A. (2003) Carbonic anhydrase inhibitors. *Med Res Rev.*; **23**: 146–89.

Takakuwa T, Konno T, Meguro H. (1985) A new standard substance for calibration of circular dichroism: Ammonium d-10-camphorsulfonate. *Anal Sci.*; **1**: 215–8.

Wong KP, Hamlin LM. (1974) Acid denaturation of bovine carbonic anhydrase B. *Biochemistry.*; **13**: 2678–82.

Yang JT, Wu CSC, Martinez HM. (1986) Calculation of protein conformation from circular dichroism. *Meth Enzymol.*; **130**: 208–78.