PEA

CARBONIC

ANHYDRASE

a kinetic study

by

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ABSTRACT

Pea carbonic anhydrase, a kinetic study

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The enzyme carbonic anhydrase (CA), catalysing the interconversion between CO_2 and HCO_3^-, has long been known to be present in plants as well as in animals. Several of the animal isozymes, but none of the plant CAs, have been extensively studied. When the first plant CA cDNA sequences were published in 1990, it was obvious that the animal and plant CAs represent evolutionarily distinct families with no significant sequence homology between the families.

Pea CA is synthesised as a precursor and subsequently processed at the import into the chloroplast. When we purified CA from pea leaves two oligomeric forms with molecular masses around 230 kDa were obtained. One form was homogenous while the other form contained subunits of two different sizes. The larger subunit has an acidic and highly charged N-terminal extension, consisting of 37 residues. We propose that the sequence that precedes the cleavage site resulting in the large subunit represents the functional transit peptide, directing CA to the chloroplast. Neither the transit peptide nor the acidic 37-residue peptide were found to affect the folding, activity or oligomerisation of pea CA.

Kinetic investigations showed that pea CA requires a reduced environment and high concentrations of buffer for maximal catalytic activity. High buffer concentrations result in a faster turnover of the enzyme (k_{cat}) while the efficiency (k_{cat}/K_m) is not affected. This is consistent with a ping-pong mechanism with the buffer as the second substrate. Both k_{cat} and k_{cat}/K_m increase with pH but the dependences cannot be described by simple titration curves. SCN^- is an uncompetitive inhibitor at high pH and a noncompetitive inhibitor at neutral and low pH. This is in accordance with the mechanistic model, previously proposed for human CAII, involving a zinc-bound water molecule as a catalytic group. In this model, the carbon dioxide - bicarbonate interconversion, reflected by k_{cat}/K_m, is temporally separated from a rate limiting proton-transfer step. At high pH, solvent hydrogen isotope effects obtained for pea CA agree with this scheme, while they do not fit at neutral and low pH.

Site-specific mutations of cysteine residues at positions 165, 269 and 272 were difficult to study, either because strong deviations from Michaelis-Menten kinetics were observed, or because the mutants were found in inclusion bodies. However, the mutant H208A was found to be a very efficient enzyme with the highest k_{cat}/K_m value obtained for any CA so far, $2.9 \cdot 10^8$ $M^{-1}s^{-1}$. With the H208A mutant an increased dependence on high buffer concentrations at low pH was obtained. At high pH, the mutant is more efficient than the unmutated enzyme. The H208A mutant is also more prone to oxidation than the wild-type enzyme.

Keywords: *Pisum sativum*, carbonic anhydrase, kinetic studies, site-directed mutagenisis.

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III. Johansson, I.-M. & Forsman, C. (1994) Solvent hydrogen isotope effects and anion inhibition of CO_2 hydration catalysed by carbonic anhydrase from *Pisum sativum. Eur. J. Biochem.,* in press

IV. Björkbacka, H., Johansson, I.-M. & Forsman, C. (1994) Studies of some site-specific mutants of pea CA. Manuscript.

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ABBREVIATIONS

CA	carbonic anhydrase
BCA	bovine carbonic anhydrase
HCA	human carbonic anhydrase
PCAt	pea carbonic anhydrase with transit peptide
PCAl	pea carbonic anhydrase, large subunit (28.2 kDa)
PCAs	pea carbonic anhydrase, small subunit (24.2 kDa)
Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase
PEPcase	phosphoenolpyruvate carboxylase
C_i	inorganic carbon
cTP	chloroplast transit peptide
E. coli	*Escherichia coli*
CD	circular dichroism
PCR	polymerase chain reaction
SDS-PAGE	sodium dodesylsulfate polyacrylamide gel electrophoresis
GuHCl	guanidinium hydrochlorid
XnumberY	site specific mutant of PCAs. X and Y are one letter codes for the amino acid that is taken away and the amino acid that is introduced, respectively. "number" is the sequence position of the changed amino acid.

INTRODUCTION

1. The aim of this investigation

Carbonic anhydrase (CA, EC 4.2.1.1, carbonate hydro-lyase) is a ubiquitous zinc-containing enzyme catalysing the reversible hydration of carbon dioxide.

$$CO_2 + H_2O \overset{CA}{\leftrightarrow} HCO_3^- + H^+$$

CA is present in both procaryotic and eucaryotic cells and in animals as well as in plants. CAs from animals are well studied. One reason for this interest is the high catalytic efficiency of the most studied isozyme, human CA II (HCA II). This enzyme has a turnover number, k_{cat}, for CO_2 hydration of $1 \cdot 10^6$ s^{-1} at pH 9 and 25°C (Khalifah, 1971). The catalytic mechanism has been elucidated, and X-ray structures have been determined for several animal CA isozymes (see 3.1 p 12 and 3.3 p 16-17).

Recently, it has been found that there are two distinct groups of CAs, α and β. The α-CA group contains all known animal isozymes and the periplasmic CA from the unicellular green alga *Chlamydomonas reinhardtii* (Tashian, 1992). The β-CA group includes CA from higher plants and from certain bacteria (Tashian, 1992). There is no sequence homology between α-CAs and β-CAs (see 3.1 p 12 and 3.2 p 13-14). It thus seems as if CA has appeared twice during evolution. Still, the α- and β-CAs catalyse the same reaction with equally high efficiency, and they are inhibited by the same kind of inhibitors (reviewed by Reed and Graham, 1981). No three-dimensional structure of a member of the β-CA group has yet been determined, and the catalytic mechanism had not been studied in detail before the start of this investigation.

To improve our understanding of the catalytic properties of a β-CA we initiated this kinetic study of CA from *Pisum sativum.* Pea was choosen as the source of plant CA for several reasons. First, the pea plants are easy to cultivate and grow rapidly. Second, like leaves of other dicotyledonous C_3 plants pea leaves contain large quantities of CA. Third, a cDNA library made from mRNA purified from light-grown plants was commercially available.

The first part of this thesis will deal with the CA history, concentrating on CA from the vegetable kingdom but also including some relevant information about animal CA, mainly HCA II. The second part is a summary of the properties we have found pea CA to possess, and it will end with some of the questions about pea CA that are left to be answered in the future.

2. Carbonic anhydrase in photosynthetic organisms

2.1 Historical background

The enzyme carbonic anhydrase has been known since 1932, when Meldrum and Roughton from red blood cells isolated an enzyme catalysing the interconversion between carbon dioxide and bicarbonate. Four years earlier, in 1928, Henriques had observed that the release of carbon dioxide from hemolysed blood was faster than the spontaneous conversion of bicarbonate to carbon dioxide. Since CO_2 is an essential component of photosynthesis, CA was also proposed to be present in plants. Several workers tried to demonstrate the existence of CA in green plants but were not successful (Roughton, 1934; Burr, 1936) until Neish, in 1939, reported CA activity in whole leaves as well as in chloroplasts of several plants. Still there were some doubts about the existence of CA in plants (Mommaerts, 1940; Day and Franklin, 1946), but in 1947 Bradfield rediscovered plant CA activity when using a sulfhydryl protecting agent in the solvent. CA activity was shown to be present in all cultivated *Brassicas*, peach, cucumber, grapefruit, spinach, lettuce, potato, sugar beet, etc.

Keilin and Mann (1939; 1940) discovered that mammalian CA contains stoichiometric quantities of zinc and that the metal ion is essential for catalysis. This was the first time a specific biological function was attributed to the zinc ion. When people started to look for zinc in plant CAs contradictory results were obtained. There were several reports stating that plant CA does not contain zinc (Kondo et al., 1952; Fellner, 1963; Rossi et al., 1969). However, in 1969 Tobin was able to purify CA from parsley, and the enzyme was found to contain one zinc ion per subunit. All subsequent reports agree that plant CAs are zinc enzymes (Kisiel and Graf, 1972; Atkins et al. 1972b; Walk and Metzner, 1975; Komarova and Doman, 1981). The

plant enzymes were also found to be inhibited by anions and sulphonamides, the same kinds of compounds that inhibit animal CAs (Bradfield, 1947; Waygood and Clendenning, 1950).

Early in the plant CA history some features distinguishing the plant enzymes from the animal CAs were observed. The most striking one was that a reducing agent was needed for plant CAs to be active (Bradfield, 1947), while this was not the case with animal CAs. Plant CAs were also found to be less sensitive to inhibition by sulphonamides (Day and Franklin, 1946; Bradfield, 1947) than animal CAs (Mann and Keilin, 1940). Animal CAs are versatile catalysts that, in addition to the reversible hydration of CO_2, can catalyse the reversible hydration of certain aldehydes and the hydrolysis of esters (reviewed by Pocker and Sarkanen, 1978). On the other hand, ester hydrolysis is not catalysed by plant CAs while there are a few reports about a weak aldehyde hydration activity (Rossi et al., 1969; Tobin, 1969 and 1970; Kisiel and Graf, 1972). The plant enzymes were also found to be oligomeric (Tobin, 1969 and 1970; Atkins et al., 1972b), while the animal isozymes are monomers.

2.2 Distribution

CA is present in almost all kinds of photosynthetic cells. CA activity has been demonstrated in both dicotyledons and monocotyledons, in plants having C_3- and C_4-type of photosynthesis as well as Crassulacean acid metabolism (CAM) and in both terrestrial and submersed plants (reviewed by Reed and Graham, 1981). CA activity is also present in unicellular as well as multicellular algae from both fresh water and marine environments (Bradfield, 1947; Bowes, 1969). Several species of cyanobacteria (Ingle and Colman, 1975), *Prochloron* sp. (Dionisio-Sese et al., 1993) and purple sulphur and nonsulphur bacteria (Ivanovskii and Rodova, 1977; Gill et al., 1984) are examples of photosynthetic eubacteria possessing CA activity.

In higher plants CA activity is found in the green parts while no activity is found in the root (Bradfield, 1947; Kisiel and Graf, 1972; Atkins, 1974; Majeau and Coleman, 1994) or in etiolated tissue (Tsuzuki et al., 1985). However, there are some reports about low CA activity even in white parts of plants and in etiolated plants (Waygood and Clendenning, 1950; Reed, 1979). Northern analysis with cDNA encoding chloroplast CA and total leaf RNA, extracted from light-grown leaves of dicotyledonous C_3 plants, shows a single hybridizing CA transcript. No hybridizing CA transcript was present when RNA was purified from etiolated leaves or root tissue (Burnell et al., 1990; Majeau and Coleman, 1991; Majeau and Coleman, 1992). Furthermore, antibodies against chloroplastic CA do not cross-react with any protein in etiolated tissue (Majeau and Coleman, 1991; Forsman, C., unpublished results). Thus, if CA is expressed in non-green parts of these plants it must be a structurally distinct isozyme.

Carbonic anhydrases from higher plants can be divided into two groups, depending on the molecular mass of the oligomeric enzymes (Atkins et al., 1972a). The high-molecular-mass group contains CA from dicotyledonous plants. The masses of native enzymes from this group have been reported to vary between 140 and 250 kDa, while the mass of the subunit is around 25 kDa. The native enzyme is thought to be a hexamer composed of identical subunits, each binding one zinc ion (reviewed by Reed and Graham, 1981, see also 5.1 p 23). The low-molecular-mass CA, 42-45 kDa, is found in monocotyledonous plants (Reed and Graham, 1981). The mass of the subunit and the zinc content have been found to be 27 kDa and 0.8 zinc ion per subunit, respectively (Atkins et al., 1972a).

The two groups are not immunologically distict since antibodies against maize CA, a C_4 monocotyledon, show cross-reactivity with all examined species (C_3 and C_4 plants, mono- as well as dicotyledons from both groups; Burnell, 1990). Antibodies against spinach CA, a C_3 dicotyledon, have been shown to cross-react

with all kinds of plants except the C_4 monocotyledons (Okabe et al., 1984). The anti-spinach CA antibodies also show cross-reactivity with two protein bands (analysed by SDS-PAGE followed by immunoblotting) from *Prochloron* sp, an unicellular symbiont that exhibits the C_3 pathway of photosynthesis and shares many features with cyanobacteria as well as with chloroplasts (Dionisio-Sese, et al., 1993).

2.3 Physiological function, intracellular location and regulation

Many efforts have been made to show that CA is required for photosynthesis, but none has succeeded so far. For instance, zinc-deficient plants possessing only a marginal CA activity (0-13%, compared to normal plants) still show high rates of photosynthesis (Randall and Bouma, 1973; Ohki, 1976). More recently Price et al. (1994) have used the antisense RNA technique to repress the CA activity in transgenic tobacco plants. Plants with CA activity levels as low as 2% of the wild-type did not have a measureable altered rate of net CO_2 fixation, and no morphological changes were observed. The major presumed functions for CA in photosynthetic organisms are: (1) rapid dehydration of stored HCO_3^- to supply substrate for enzymes using CO_2 as substrate (e.g. ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)), (2) rapid hydration of CO_2 to capture inorganic carbon within the cell or cell compartment, or for use of the produced HCO_3^- as substrate for some enzymes (e.g. phosphoenolpyruvate carboxylase (PEPcase)), (3) facilitation of inorganic carbon transport whithin or into the cell or cell compartment and (4) pH regulation at sudden pH jumps (reviewed by Reed and Graham, 1981; Sültenmeyer et al., 1993).

The localisation of CA in plants has been under debate. Isozymes with different locations seem to be present. The first report about putative isozymes came after polyacrylamide gradient gel electrophoresis of whole leaf extracts, followed by CA activity staining. More than one protein band with CA activity was found in all tested

plant species (Atkins et al., 1972b; Reed, 1979). In green leaves from dicotyledonous C_3 plants two high-M_r bands, and sometimes one low-M_r band, with CA activity were observed. The larger of the two high M_r bands was absent from partially purified chloroplasts (Graham et al, 1974; Walk and Metzner, 1975; Reed, 1979). There are also reports about purified isozymes from pea and bean leaves but the information about these isozymes is very scanty. At least one of them seems to be localised in the chloroplast, however (Kachru and Anderson, 1974; Komarova and Doman, 1983). Other suggested locations of isozymes include the chloroplast surface, thylakoid membrane, plasma membrane and cytosol (Everson, 1970; Stemler, 1986; Moubarak-Milad and Stemler, 1994; Utsunomiya and Muto,1993; Reed, 1979).

The location of the main CA isozyme depends on the type of photosynthesis used by the plant. In terrestrial dicotyledonous C_3 plants fixing CO_2 by Rubisco in the chloroplasts, CA is found as a soluble enzyme in the chloroplast stroma (Everson and Slack, 1968; Poincelot, 1972; Jacobson et al., 1975). CA is in fact one of the most abundant chloroplastic enzymes, representing 1-2 % of the total soluble leaf protein (Tobin, 1969 and 1970; Okabe et al., 1984). Price et al. (1994) concluded from repression experiments that the physiological function of the enzyme is to facilitate transfer of CO_2 within the chloroplast, producing a marginal improvement in the efficiency of photosynthesis. But they also point out that the CA activity left in the cells during their experiments (2%) may be playing some yet unexplained role.

The initial carboxylation reaction in C_4 photosynthesis is catalysed by PEPcase, which utilizes bicarbonate rather than CO_2 as substrate (O´Leary, 1982). The main, or only, location of CA in C_4 plants is the same as the location of PEPcase, the cytosol of the mesophyll cells (Everson and Slack, 1968; Burnell and Hatch, 1988). CA may then be regarded as catalysing the first step in C_4

photosynthesis. This is the rapid conversion to bicarbonate of the atmospheric CO_2 entering the mesophyll cells (Hatch and Burnell, 1990). There are reports claiming that the C_4 CA is membrane attached (Atkins et al., 1972a; Utsunomiya and Muto, 1993; Stemler, 1986; Moubarak-Milad and Stemler, 1994).

Efforts have been made to find some environmental regulation of the plant CA activity. Proposed regulators are light as well as environmental CO_2 concentrations. Recently, the 5′ flanking region of the gene encoding chloroplastic CA in *Arabidopsis thaliana* was found to contain sequences that are homologous to motifs that play roles in the tissue-specific and light-modulated expression of the small subunit of Rubisco (Kim et al., 1994). The regulation of CA activity in algae and cyanobacteria has been more extensively studied. In these organisms CA is found to be induced by growth at low CO_2 concentrations, and at the same time an active mechanism for accumulation of inorganic carbon (C_i) is induced. In the cyanobacterium *Synechococcus*, CA is found to be associated with the carboxysomes (Price et al., 1992). These are small polyhedral bodies surrounded by a thin protein shell (Codd and Marsden, 1984) and containing the majority of the Rubisco activity. There are also indications of the presence of a non-carboxysomal CA (Price et al., 1992). Both CA activities have been shown to increase when *Synechococcus* cells are adapting to low C_i environment (cultures gassed with air containing 350 ppm CO_2 or less; Price et al., 1992). From *Synechococcus* PCC7942 the gene *icfA,* coding for a β-CA, has been isolated as a genomic region complementing a temperature-sensitive and high-CO_2-requiring mutant (Fukuzawa et al., 1992; Yu et al., 1992). In studies of the *icfA* mutant the carboxysomal CA was found to be essential for CO_2 fixation but not for the accumulation of C_i (Fukuzawa et al., 1992). The most studied algal CA is from the unicellular green alga *Chlamydomonas reinhardtii.* Most of the CA activity in this species is found to be extracellular, located in the periplasmic space (Kimpel et al., 1983). Both reduction of the C_i level and light is needed for induction of this extracellular CA (Nelson et al., 1969; Spalding and Ogren, 1982; Spencer et al.,

1983). The increase in enzyme activity is due to an increase in the amount of CA oligomer, and is found to be regulated at a level prior to translation (Coleman and Grossman, 1984; Toguri et al., 1984). Accumulation of CA mRNA has been found to require photosynthesis (Fukuzawa et al., 1990a). The presence of external CA in algae seems to correlate with the ability to import external HCO_3^- into the cell.

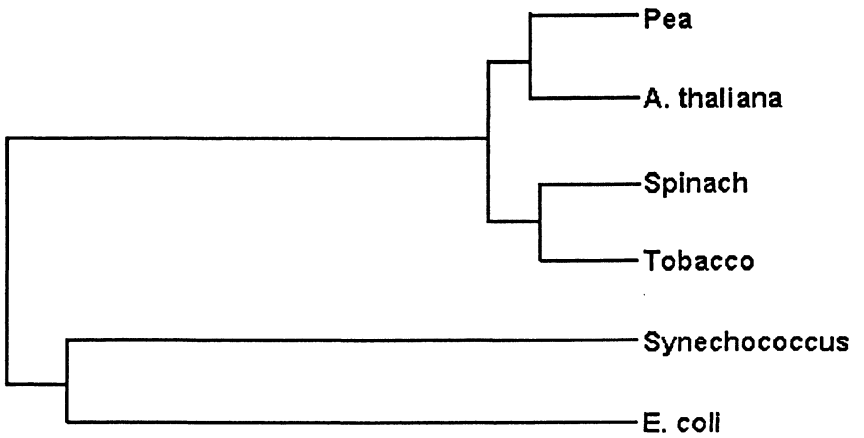

Fig. 1 Generalized phylogenetic branching scheme for the β-CAs

3. Plant CA in comparison with animal CA

3.1 Enzyme structure, α-CA

In mammals seven different CA isozymes are known, each with characteristic properties and cellular distribution (Tashian, 1992). The primary structures of all sequenced animal CAs are found to be homologous with several conserved regions (Venta et al., 1987). The three-dimensional structures of three cytoplasmic mammalian isozymes, human CA I and II (HCA I and II) and bovine CA III (BCA III), have been solved by X-ray crystallography at 0.2-nm resolution or higher (Kannan et al., 1984; Eriksson et al., 1988; Håkansson et al., 1992; Eriksson and Liljas, 1993). These isozymes are monomers with molecular masses around 29 kDa, and the folding of the polypeptide chains is very similar. The active site is in the form of a conical cavity with the zinc ion at the bottom near the centre of the molecule. The zinc ion is coordinated to three conserved histidine residues and a water molecule, that can ionise to a hydroxide ion, giving an almost regular tetrahedral geometry around the zinc ion. A network of hydrogen bonds, including bridging water molecules, connects the metal ion to a number of conserved active site residues. This network has been found to be important for catalysis (Liang et al., 1993; Xue et al., 1993), and a proposed function is to restrict the orientation of the zinc-bound hydroxide (Merz, 1990).

The first CAs to be cloned from a photosynthetic species were two periplasmic isozymes from the unicellular green alga *Chlamydomonas reinhardtii* (Fukuzawa et al., 1990b). The derived amino acid sequences show 91.8 % identity and were shown to be about 20 % identical to known mammalian isozymes. The conserved zinc ligands were found to be present together with most of the amino acid residues taking part in the active site hydrogen bonding network as well as some other invariant positions (Fukuzawa et al. 1990a).The periplasmic *C. reinhardtii* CA

enzymes have been shown to be heterotetramers composed of two large subunits (35 kDa) and two small subunits (4 kDa) linked by disulfide bonds (Kamo et al., 1990; Tachiki et al., 1992; Ishida et al., 1993). The large subunit has been shown to bind zinc (Kamo et al., 1990), and to be N-glycosylated at three positions (Ishida et al., 1993). However, there are also reports about oligomeric CA forms containing up to three large subunits and at least two small subunits (Husic, 1990; Husic et al., 1991). The cDNA sequence indicates that the two different subunits are cotranslated as a large precursor consisting of the signal peptide, the large subunit, 35 intervening amino acids and the small subunit (Fukuzawa et al., 1990b; Ishida et al., 1993). Nothing is known about the three dimensional structure of these two isozymes.

3.2 Enzyme structure, β-CA

The first published higher-plant CA sequence was a 20 amino acid sequence from a cyanogen bromide fragment of spinach CA (Hewett-Emmett et al., 1984). This sequence showed no clear homology with the mammalian isozymes. Indeed, when the cDNA encoding the spinach chloroplastic CA was cloned and sequenced the derived amino acid sequence showed no homology at all with known mammalian CAs (Burnell et al., 1990; Fawcett et al., 1990). The main location of CA in C_3 plants is the chloroplasts (see 2.3, p 9), while the enzyme is coded by a gene on a nuclear chromosome. The enzyme is thus synthesised in the cytoplasm with an N-terminal extention, the chloroplast transit peptide (cTP), that is subsequently cleaved off during or after the import into the chloroplast. The open reading frame of spinach CA was shown to encode a protein of 319 amino acids, giving a molecular mass of 34.6 kDa. N-terminal sequencing of CA purified from spinach leaves was used to identify the cleavage site of the transit peptide. The mature enzyme was found to consist of 221 amino acids, giving a subunit molecular mass of 24.2 kDa. However, Fawcett et al. (1990) reported that CA appeared to be proteolyzed during purification since they

found newly imported CA to have an apparent molecular mass close to 30 kDa. No evidence for this newly imported form of CA was presented. They also claimed that the 9 acidic residues between amino acid 60 and 100 were unlikely to be part of a transit peptide, proposing that, approximately, the first 60 amino acids constitute the transit peptide. (See 5.1 p. 23)

Four additional plant CA cDNA sequences have been published: pea (Roeske and Ogren, 1990; Majeau and Coleman, 1991), tobacco (Majeau and Coleman, 1992) and two *Arabidopsis thaliana* sequences (Raines et al., 1992; Palma-Fett and Coleman, 1994). From *Arabidopsis thaliana* the nucleotide sequence of the chloroplast CA gene is also known (Kim et al., 1994). The β-CA group has also been found to include CA from *E. coli* (Sung and Fuchs, 1988; Guilloton et al., 1992) and from the cyanobacterium *Synechococcus* (Fukuzawa et al., 1992; see also 2.3, p 10). The higher plant CAs show a high degree of identity, 61 % when only the mature enzymes are taken into account (from amino acid 108 in the pea CA sequence, Fig. 2). The two *Arabidopsis thaliana* CA sequences are 87 % identical (the mature part). The two procaryotic β-CAs are 20-30 % identical to the mature form of the higher plant CAs. Fig. 2 shows the deduced amino acid sequence for pea CA. Amino acids that are conserved among the five sequenced plant enzymes are shown in bold letters, while the positions that are conserved in all seven β-CAs are marked beneath by a *. Zinc binding candidates are indicated by a ♦. In accordance with the α-CAs it is reasonable to assume that the zinc ion present in β-CAs is catalytically functional. Zinc ions that are directly acting in catalytical events have been found to be coordinated to three protein ligands and one "activated" water molecule (Vallee and Auld, 1989). Histidine is by far the most common amino acid ligand but cysteine, glutamic acid and aspartic acid are also found. In zinc enzymes with known X-ray structures two of the ligands have been found to be spaced by only 1-3 amino acids while the third ligand is relatively distant (17-122 amino acids). By looking at the amino acids that are conserved in all β-CAs six possible zinc binding

candidates are found. These are Cys159, Asp161, Glu203, His219, Cys222 and Glu275. If any of these bind to the zinc ion is presently unknown.

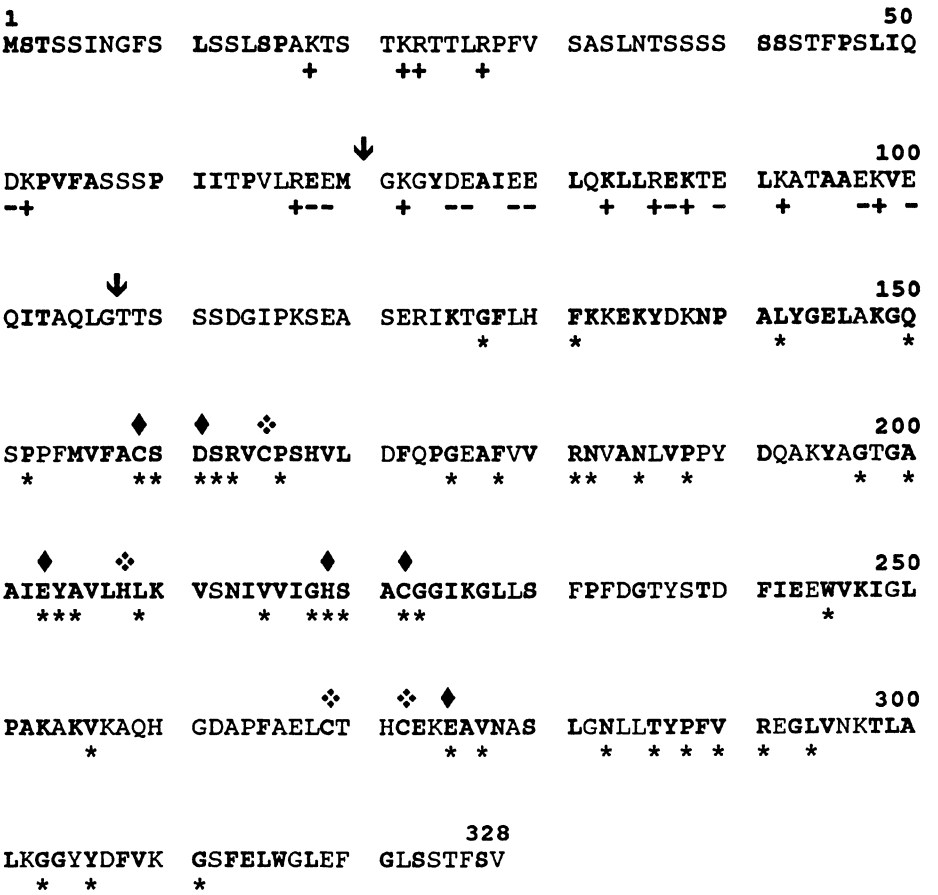

Fig. 2 The pea CA amino acid sequence, deduced from the cDNA sequence. Amino acids that are conserved among the five known plant sequences are shown in bold letters. Amino acids that are conserved in all β-CAs are marked by *. The charges of the cTP residues are shown beneath them. Candidates for zinc binding (♦), positions that we have changed by site-directed mutagenesis (❖) and the two identified processing sites (🡻) are also shown (see sections 5.1 and 5.4).

The only structural information, beyond the amino acid sequences and the fact that higher plant CAs are oligomers, has come from optical rotatory dispersion and circular dichroism (CD) spectra for the parsley enzyme (see also 5.2, p 25). The content of α-helix was from the optical rotatory dispersion spectra calculated to be 27% (Tobin, 1970). However, such theoretical calculations of secondary structures are uncertain. There have been speculations that the plant CA active site, or the funnel into the active site, might be smaller than the one in animal CAs (Pocker and Ng, 1974). However, the only reasons for this suggestion were the weaker inhibition by sulphonamides and the lower versatility in catalysed reactions (see 2.1 p 6). There is presently no structural information about the active site.

3.3 Catalytic mechanism

The enzymatic mechanism of HCA II has been extensively studied. This is one of the most efficient enzymes known, having a turnover number, k_{cat}, for CO_2-hydration of $1 \cdot 10^6$ s^{-1} at 25°C and pH 9 (Khalifah, 1971). The catalytic reaction has been shown to consist of two parts (see Silverman and Lindskog, 1988, for a review). The first part is the interconversion between CO_2 and HCO_3^- (Eqn 1) and is reflected by k_{cat}/K_m. This part of the reaction is not sensitive to the buffer concentration and exhibits no solvent hydrogen isotope effect.

$$(1)\quad E\text{-}Zn^{2+}\text{-}OH^- + CO_2 \longleftrightarrow E\text{-}Zn^{2+}\text{-}HCO_3^- \overset{H_2O}{\longleftrightarrow} E\text{-}Zn^{2+}\text{-}H_2O + HCO_3^-$$

$$(2)\quad E\text{-}Zn^{2+}\text{-}H_2O \longleftrightarrow {}^{+}H\text{-}E\text{-}Zn^{2+}\text{-}OH^- \overset{B}{\underset{BH^+}{\longleftrightarrow}} E\text{-}Zn^{2+}\text{-}OH^-$$

The second part (Eqn 2) is the regeneration of the active form of the enzyme. This part of the reaction involves the transfer of a proton between the zinc-bound water molecule and the surrounding medium. In HCA II the proton is transferred in two

steps, shuttled via amino acid residue His64 from the active site to a buffer molecule. At high buffer concentrations the intramolecular step (first part of Eqn 2) is rate-limiting, while at low buffer concentrations the proton transfer from His64 to the buffer molecule is rate determining (second part of Eqn 2). Eqn 2 is reflected by the kinetic parameter k_{cat} since it is always the rate determining part of the catalytic cycle. In agreement with Eqn 2, k_{cat} has been shown to be dependent on the buffer concentration and to exhibit a solvent hydrogen isotope effect of 3.8 (Steiner et al., 1975). The pH profiles for k_{cat} and k_{cat}/K_m both follow simple titration curves with pK_a values close to 7. In the case of k_{cat}/K_m this reflects the titration of the zinc-bound water molecule, the catalytically active group, which can ionise to a hydroxide ion. In the CO_2-hydration reaction the basic form of this group is active while the reverse reaction needs the protonated form. The pH profile of k_{cat} reflects the titration of the proton-shuttle group, His64. The mechanisms of other isozymes have not been as simple to elucidate. BCA III is a much slower enzyme than HCA II, k_{cat} is around $3 \cdot 10^3$ s^{-1} for CO_2 hydration and is almost constant in the pH range 5.5 to 8. The mechanism has been suggested to be the same as for HCA II, with a rate-limiting proton transfer. However, there is no evidence for any intramolecular proton transfer (Ren et al., 1988). In the case of the intermediately active HCA I, possessing a k_{cat} value for CO_2 hydration of $2 \cdot 10^5$ s^{-1} at high pH (Khalifah, 1971), both proton transfer and dissociation of HCO_3^- seem to contribute to the rate limitation (Lindskog et al., 1984; Behravan et al., 1990).

The kinetic properties of plant CAs have been studied to a limited extent only, and the catalytic mechanism has not been elucidated. As pointed out already in 1947 the activity of most plant CAs depend on a reduced environment (Bradfield, 1947). This has later been confirmed for purified CA from spinach, parsley, cotton and pea (Rossi et al., 1969; Tobin, 1970; Chang, 1975; Atkins et al., 1972; 5.3 p 25). In contrast to all others working with CA from spinach Pocker and Ng (1973) reported that the enzyme activity does not depend on the presence of sulfhydryl

reducing agents. Plant CAs have been found to be inhibited by chemicals such as iodoacetate and dithionitrobensoic acid (DTNB) which react covalently with free SH groups. This has been interpreted as a requirement of free SH groups for the catalytic activity of plant CAs (Rossi et al.,1969; Tobin, 1971; Chang, 1975; Kandel et al., 1978; Cybulsky et al., 1979).

Tobin (1970) made the first kinetic study of a plant CA, from parsley, using a stopped-flow spectrophotometer and the changing-pH/indicator method. He found the kinetic parameter k_{cat} for CO_2 hydration to be $1 \cdot 10^5$ s^{-1} per subunit at high pH and 25°C, in the presence of a reducing agent. A more extensive study has been made of the enzyme purified from spinach (Pocker and Ng, 1973; Pocker and Ng, 1974; Pocker and Miksch, 1978). Since all their activity measurements were done without any reducing agent present and at high concentrations of both chloride and sulfate, potent anionic inhibitors of plant CA (see 5.3 p 25), the kinetic parameters obtained are not comparable with those from measurements without inhibitors present and with the use of fully active (reduced) plant enzymes.

PRESENT WORK

4. Methodology

4.1 Cloning of the pea CA cDNA

A convenient way to amplify a known DNA sequence is to use the PCR (polymerase chain reaction) technique. We have used this method to amplify the pea CA cDNA from a pea cDNA library, with the purpose to clone and express pea CA in *E. coli* (see 5.2 p. 24). PCR takes advantage of a heat stable DNA polymerase originating from thermophilic bacteria. The method involves an automatic cycling through (1) heat denaturation producing single-stranded DNA (2) binding of DNA oligonucleotides (primers) that are complementary to the sequence at both ends of the piece of DNA to be amplified (primer annealing) and (3) extension of the primers by the DNA polymerase. Each cycle will duplicate the DNA of interest. Cleavage sites for restriction enzymes were introduced on each side of the coding region during the PCR by allowing for some mismatched bases within the primers. The amplified DNA was purified, cleaved at the introduced restriction sites, and cloned into the mutagenisis/expression vector pACA (Nair et al., 1991), replacing the HCA II gene.

The plasmid pACA contains a f1 origin of replication and confers ampicillin resistance. The pea cDNA was placed under control of the T7 RNA polymerase promoter (Studier and Moffatt, 1986). For production of pea CA the *E. coli* cells BL21(DE3), containing a single copy of the T7 RNA polymerase gene in the chromosome under control of the inducible *lac* UV5 promoter, were used.

4.2 Site-directed mutagenesis

Site-directed mutagenesis is a helpful tool in studies of the catalytic mechanisms of enzymes. The mutagenesis method used in this work was the "Kunkel method" (Kunkel, 1985). In this method the *E.coli* strain CJ236, which is deficient in the enzymes uracil N-glycosylase (ung^-) and dUTPase (dut^-), is used to produce a single stranded DNA template containing deoxyuridine instead of deoxythymidine. The mutagenesis is done *in vitro* using a phosphorylated DNA oligonucleotide with the desired base changes. Chain elongation is done in the presence of dTTP. The resulting double-stranded plasmid is a heteroduplex with a uracil-containing parent strand and a thymidine-containing mutant strand. Introduction of this plasmid into a "wild-type" *E.coli* strain (dut^+,ung^+) will give rise to mostly mutant progeny, due to the strong biological selection against the uracil-containing parental strand. All produced mutants were checked by sequencing the whole pea CA coding region.

4.3 Kinetic measurements

To measure the catalytic efficiency of pea CA under different steady-state conditions, the changing-pH/indicator method was used. In this method pairs of buffers and indicators with nearly the same pK_a values are used. The change in absorbance during the proton-producing reaction was followed with a stopped-flow spectrophotometer (Khalifah, 1971; Steiner et al., 1975). The initial rate of CO_2 hydration was computer calculated as

$$-| A_0-A_{eq} |\cdot(d \ln |A-A_{eq}| / dt)_0$$

were A_0 and A_{eq} are initial and equilibrium absorbance values, respectively.

The kinetic parameters k_{cat} and K_m were calculated by fitting the rate data to the Michaelis-Menten equation (see below) by non-linear regression, using the program GraFit (Erithacus Software Ltd, UK).

$$v_0 = k_{cat} [E]_0 [CO_2] / (K_m + [CO_2])$$

k_{cat} , the turnover number, is a function of all first-order rate constants and will reflect the rate-limiting step in the reaction. The ratio k_{cat}/K_m is an apparent second order rate constant describing the catalysis at substrate concentrations well below K_m. In a sequential mechanism the parameter k_{cat}/K_m will include rate constants from the encounter of the substrate with the enzyme through the first irreversible step, in this case the dissociation of HCO_3^-.

Solvent hydrogen isotope effects, using H_2O and D_2O as solvents, can be used to determine if proton transfer is rate limiting in a chemical reaction. The transfer rate of an exchangeable proton is usually 2-7 times smaller in D_2O than in H_2O. This is because the deuteron is more tightly bound to the molecule and the activation energy needed for the transfer of deuterium, therefore, is higher than for the transfer of hydrogen. Another effect of the changed binding is that an acid become weaker in D_2O than in H_2O, resulting in an increased pK_a value of about 0.4 pH units. However, for residues in an active site the solvent effect might be variable. Deuterones in enzymes might also give rise to small but significant structural changes.

5. Presentation of papers I - IV

5.1 Purification of CA from pea leaves and identification of cleavage sites within the cTP (papers I and II)

With the aim to learn more about the catalytic mechanism of a plant CA, this project started with laborious efforts to find a fast and simple purification method for the pea enzyme. The solution to the problem was the use of affinity chromatography with p-aminomethylbenzene sulphonamide coupled to epoxy-activated Sepharose 6B (Pharmacia). The attachment site for the ligand is at the end of a 14-atom long spacer arm. Even though plant CAs are inhibited fairly poorly by sulphonamides (50 % inhibition by 28 μM acetazolamide), this affinity method was found to be effective. From 600 g of plant material around 20 mg of pure pea CA was obtained in a single purification step. Later, we have found that pea CA does not bind to the affinity gel commonly used to purify mammalian CAs. The ligand is the same but there is no spacer arm. Instead, the sulphonamide is directly coupled to carboxymethyl groups on the matrix (Khalifah et al., 1977).

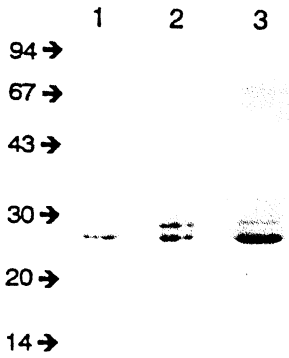

Fig. 3 SDS-PAGE analysis of pea CA purified from pea leaves. Bands excised from native PAGE were run in lane 1 (lower band) and lane 2 (upper band). Lane 3, CA purified from pea leaves.

Purified pea CA appears as a doublet on non-denaturing as well as denaturing polyacrylamide gels (Fig. 3, lane 3). The major oligomeric form was found to be homogeneous, only consisting of 25 kDa subunits (from SDS-PAGE). The upper band from the nondenaturing gel was found to represent a heterooligomer composed of equal amounts of 25 and 27 kDa subunits (Fig. 3, lane 1 and 2). From both electrophoresis and gel-filtration chromatography a molecular mass around 230 kDa was obtained for the oligomers. Thus, our results indicate that pea CA is an octamer, while most authors have proposed that CA from pea and spinach is a hexamer (Reed and Graham, 1981). A definite answer about the quaternary structure of pea CA will probably not be found until the crystal structure has been solved.

N-terminal sequencing and comparison with the published pea CA sequence identified the two cleavage sites that give rise to the two subunits (Fig. 2, p 15). Calculated from the sequence, the M_r values of the small and large subunits are 24 200 and 28 200, respectively. The 37-residue sequence between the two cleavage sites is highly charged with a net charge of -2. From secondary structure predictions an α-helix was found to be the most probable structure. In a helical wheel representation all acidic residues were found to be located on one side of the wheel while hydrophobic residues were found to dominate the other side (paper II, Fig. 3). Similar sequences are also present in the other sequenced chloroplastic CAs and, as pointed out by Fawcett et al. (1990; see 3.2 p 13), this sequence does not look like part of a cTP. A high content of serine and threonine and the presence of few, if any, negatively charged amino acids are pronounced characteristics of cTPs. Theoretical calculations have shown that the C-terminal 8-10 amino acids before the processing site have a high potential to fold into an amphiphilic β-strand (von Heijne et al., 1989). It thus seems probable that the sequence preceding cleavage site 1, which has the properties of a cTP, is the functional transit peptide. Results of import experiments with different pea CA constructs and isolated chloroplasts have also

shown that the acidic peptide, between cleavage sites 1 and 2, is not necessary for import (C. Forsman, personal communication).

5.2 Pea CA expression in *E. coli* and some characteristics of the enzyme (papers I and II)

To obtain large quantities of homogenous enzyme as well as to make site-specific mutants, we decided to clone and express the pea CA cDNA in *E. coli*. The cDNA was isolated with the PCR technique and subsequently cloned into an expression/ mutagenisis vector (see 4.1 p 19). The resulting plasmid, carrying the entire coding region of the pea CA gene, was called pPCAt. By site-directed mutagenesis followed by restriction enzyme cleavage and religation the variants pPCAl, coding for the large subunit, and pPCAs, coding for the small subunit, were obtained.

All three enzyme variants were expressed in *E. coli* as soluble enzymes that could be purified in the same way as the enzyme from the plant material. By analysing the purified enzymes with denaturing electrophoresis, PCAl and PCAs were found to be homogenous and to migrate in the same way as the large and small subunits from pea leaves, respectively. On the other hand, PCAt was partially processed in *E. coli*, and three major bands were observed. Their sizes ranged from that of enzyme with unprocessed cTP to that corresponding to the small subunit. The highest yield of enzyme, 40-60 mg of pure CA per litre of culture, was obtained with PCAs. All three enzyme variants were highly active, and the M_r values of the oligomeric enzymes were found to be 230 000 or more. Thus, neither the transit peptide nor the acidic peptide in the N-terminal of the large subunit seem to affect folding, catalytic activity or oligomerization of pea CA.

In addition to the kinetic measurements (presented in 5.3), CD spectra and the stability towards denaturation of CA purified from pea leaves as well as PCAs purified from *E. coli* were studied. No significant differences were observed between these two enzymes. However, the CD spectra were found to be quite different from that of HCA II (paper I, Fig. 2). Thus, the differences between plant CA and human CA with respect to primary as well as quaternary structures seem to extend also to the secondary structure (see 2.2, 3.1 and 3.2). The stability measurements indicated the presence of two transitions. The inactivation transition was found to appear at about 1.4 M GuHCl and coincided with a small increase in the relative fluorescence intensity (followed at 332 nm). The second transition, at 2.4 M GuHCl, was seen as a large decrease of the relative fluorescence intensity at 332 nm (paper I, Fig. 3).

5.3 Kinetic studies of pea CA (papers I and III)

We have investigated the CO_2-hydration reaction catalysed by pea CA under different conditions. Pea CA was found to have some catalytic features in common with HCA II (see 3.3, p 16-17), as well as some that differ from the characteristics of the human enzyme.

Under optimal conditions pea CA was found to be highly active. The highest observed turnover rate, k_{cat}, was $8 \cdot 10^5$ s^{-1} (in 80 mM Barbital buffer, pH 8.2) while the highest k_{cat}/K_m value was $1.8 \cdot 10^8$ $M^{-1}s^{-1}$ (in Taps pH 9.0), both values calculated with respect to the subunit. To keep the enzyme maximally active, several criteria have to be fulfilled. First of all a reducing agent must be present. Purification of pea CA without any reducing agent results in an oxidised, inactive enzyme. Second, a certain ionic strength was found to be needed. At neutral and high pH the activity increased when Na_2SO_4 was added to a total ionic strength of 50 mM. At low pH pea CA is highly sensitive to inhibition by anions, including sulphate ions, and inhibition instead of activation was obtained if Na_2SO_4 was added. Third, high

concentrations of buffer were found to be needed for pea CA to work at maximal rate. At increasing buffer concentrations the observed k_{cat} as well as K_m values increased, leaving k_{cat}/K_m essentially unchanged. This is exemplified for Taps at pH 9 in Fig. 4 (from paper I, Fig. 6A). K_m values for the buffers could be estimated by considering the buffer as a second substrate (see insert in Fig. 4). For the buffers investigated at neutral and high pH, K_m values calculated on the basis of the total buffer concentration varied between 19 and 185 mM. Since the basic form of the

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Fig. 4 Effects of the buffer concentration on the CO_2 hydration catalysed by pea CA in Taps pH 8.7. The ionic strength was kept constant at 50 mM by the addition of Na_2SO_4 at buffer concentrations below 100 mM. The buffer concentrations are 150 mM, 100 mM, 50 mM, 25 mM and 10 mM. As insert is shown a secondary plot were $[B_t]$ refers to the total buffer concentration

buffer seemed to be the active buffer species (paper I) we recalculated these K_m values to represent only the basic form of the buffer. Values between 12 and 82 mM were then obtained. With barbital as buffer the highest turnover number at infinite buffer concentration as well as the lowest K_m for the basic form of the buffer were obtained. Thus, barbital buffer functions as a better substrate than both buffers of

the biological type and imidazole derivatives. With Mes buffer at pH 6 as well as with DMI (dimethylimidazole) at pH 9, we obtained non-Michaelis-Menten kinetics, and no certain kinetic parameters were obtained. However, an activating effect of the buffer concentration could still be seen and by extrapolations from the curved plots a K_m value of 4 mM, for the total buffer concentration, was obtained for Mes buffer (paper I, Fig. 7 and paper III, Fig. 2). The dependence of the activity on the buffer concentration is in accordance with a ping-pong mechanism with the buffer as the second substrate, in analogy with the behaviour of HCA II. Much higher buffer concentrations are needed, however, for pea CA to work at a maximal rate than for HCA II (with K_m values for the total buffer concentration below 10 mM). Thus, the pea CA results fit with the catalytic mechanism proposed for HCA II, summarised in eqns. 1 and 2.

$$(1)\quad E\text{-}Zn^{2+}\text{-}OH^- + CO_2 \longleftrightarrow E\text{-}Zn^{2+}\text{-}HCO_3^- \overset{H_2O}{\longleftrightarrow} E\text{-}Zn^{2+}\text{-}H_2O + HCO_3^-$$

$$(2)\quad E\text{-}Zn^{2+}\text{-}H_2O \longleftrightarrow {}^+H\text{-}E\text{-}Zn^{2+}\text{-}OH^- \overset{B}{\underset{BH^+}{\longleftrightarrow}} E\text{-}Zn^{2+}\text{-}OH^-$$

Both k_{cat} and k_{cat}/K_m increase with pH, but do not follow simple titration curves (Fig. 5, p 31, open circles). The buffer concentration used in this study was 50 mM (25 mM at low pH). By plotting the $(k_{cat})_{max}$ values, e. i. the k_{cat} values extrapolated to infinite buffer concentration, from the buffer-dependence studies, the same kind of pH dependence is still observed. The parameter k_{cat}/K_m does not change with the buffer concentration. This complex pH dependence suggests that more than one titratable group affecs the activity of pea CA.

To further investigate the pea CA mechanism anion inhibition was studied, using the strong inhibitor SCN^- in the pH range 6-9. The anion binding was found to be pH dependent (paper III, Fig 6), with the strongest binding at low pH. But the pH

dependence cannot be described by a simple titration curve, in analogy with the pH dependence of the pea CA activity. The inhibition patterns obtained with SCN^-, uncompetitive at high pH (8.6) and noncompetitive at neutral and low pH (paper III, Fig. 3, 4 and 5), are the same as those reported for HCA II (Tibell et al., 1984). The results are in agreement with the two equations outlined above if the proton transfer steps of eqn. 2 are rate limiting and the pK_a of the activity-linked group is below pH 8.6. At pH 8.6, the unprotonated enzyme form will accumulate at substrate concentrations well below K_m (k_{cat}/K_m conditions), and there will be little or no inhibition by anions. On the other hand, at high substrate concentrations (k_{cat} conditions) the enzyme form preceding the rate limiting step will accumulate. Since this is a protonated enzyme form according to eqn. 2, a strong anion binding will be obtained, resulting in an uncompetitive inhibition pattern. At low pH, a protonated enzyme form is predicted to accumulate at low as well as at high substrate concentrations and the anion inhibition will be noncompetitive, in accordance with our observations.

If the proton transfer step is rate limiting in the pea CA mechanism and the two equations outlined above are valid, then a solvent hydrogen isotope effect larger than 2 should be found for k_{cat}, while no isotope effect should be obtained for k_{cat}/K_m. The isotope effects observed for pea CA are comparatively small, with a value around 2 for k_{cat} under all investigated conditions. The effects on k_{cat}/K_m and on K_m were found to depend on pH. At high pH the effect on K_m was at least 3, giving a value below 1 for k_{cat}/K_m, while at low pH the major effect was found for k_{cat}/K_m. It thus seems as if the HCA II mechanism is valid for pea CA at high pH, with at least partial rate limitation in the proton transfer step. At neutral and low pH the situation is different. The mechanism seems to have changed so that a proton transfer now seems to be reflected in k_{cat}/K_m, in apparent conflict with eqn. 1.

5.4 Studies of site-specific pea CA mutants (paper IV)

To further explore the properties of pea CA, site-specific mutants were made. A major drawback is the limited information available about the structures of β–CAs, implying that guesses must be made concerning the residues of interest.

However, the cysteines and their possible functions attracted our attention. Is a Cys residue part of the catalytic site, and would it be possible to decrease or eliminate the sensitivity to oxidation by replacing one or several Cys residues with non-oxidisable residues ? Five cysteines are present in each subunit of pea CA. Two of these are conserved, Cys159 and 222, while the other three are absent in the procaryotic β-CAs (Cys165, 269 and 272). Since CA from the cyanobacterium *Synechococcus*, in contrast to pea CA, has been found to loose activity in the presence of the reducing agent DTT (Price et al., 1992), cysteines 159 and 222 do not seem to be the residues responsible for the oxidation sensitivity of pea CA. Consequently, we have changed Cys165, 269 and 272 to Ser or Ala in this first study. The changed enzymes were found to be difficult to study. The change of Cys165 to Ala or Ser resulted in soluble enzymes that could be purified with the usual purification method. These mutants were found to be almost as active as the unmutated enzyme. However, strong deviations from Michaelis-Menten kinetics were obtained and no kinetic parameters could be determined (paper IV, Fig. 2). These two mutants were still found to be sensitive to oxidation. All mutations at positions 269 and 272 resulted in enzymes accumulating in the *E. coli* cells as inclusion bodies. The small fraction of soluble enzyme found in crude *E. coli* extracts was active, however. Thus, these cysteine residues might be important for the proper folding of the enzyme. It is also tempting to speculate that these two are the residues responsible for the oxidation sensitivity.

Another interesting problem is concerning the proton transfer pathway and whether there is an internal proton-transfer group in pea CA. Since His residues often have pK_a values around neutral pH, and the pH in stroma changes around 7-8, one His residue was chosen for further studies. His208 is conserved among the chloroplastic CAs and was changed to the non-ionisable residue Ala.

The first and unexpected characteristic of the H208A mutant was an enhanced sensitivity to oxidation. Purification of H208A by the same procedure as the wild-type enzyme resulted in an enzyme with low specific activity. By including a reducing agent at an early stage of the purification and by using a short time of dialysis against buffer containing TCEP (Tris(2-carboxyethyl)phosphine) instead of cysteine, the specific activity increased to the level of unmutated pea CA (Paper IV, table. 1).

In kinetic studies of CO_2 hydration we found that also the H208A mutant follows a ping-pong mechanism with the buffer as a second substrate (paper IV, Fig. 4). However, the concentration of buffer needed for the enzyme to work at maximal rate has increased at neutral and low pH. At low pH an apparent K_m value of 340 mM was obtained, with respect to the total buffer concentration, while the K_m for the unmutated enzyme was only 4 mM. A considerable higher value for the mutant than for the wild-type enzyme, despite the uncertainty in the wild-type enzyme value (see 5.3 p 27). The patterns of SCN^- inhibition were the same as those observed for the unmutated enzyme including the pH dependence. However, SCN^- was found to bind to H208A with about half the affinity of the unmutated enzyme at high pH (paper III, Fig. 6 and paper IV, Fig. 5). An interesting difference from the unmutated enzyme was found for the pH dependence of k_{cat}/K_m (Fig. 5, p 31 lower part). In contrast to the unmutated enzyme the pH dependence of the mutant could be described by a simple titration curve with a pK_a value of 7.4. At neutral and high pH H208A was found to be a more efficient catalyst of CO_2-hydration than the unmutated enzyme, while at low pH there was no significant difference. In fact, the upper limit for k_{cat}/K_m

of $2.9 \cdot 10^8$ $M^{-1}s^{-1}$ with respect to the subunit is the highest value obtained for any carbonic anhydrase so far. The pH dependence of k_{cat} (50-100 mM buffer) showed only small differences from that of the unmutated enzyme (Fig. 5, p 31 upper part). The difference observed at low pH could be attributed to the different buffer concentration dependence of the two enzymes in this pH region.

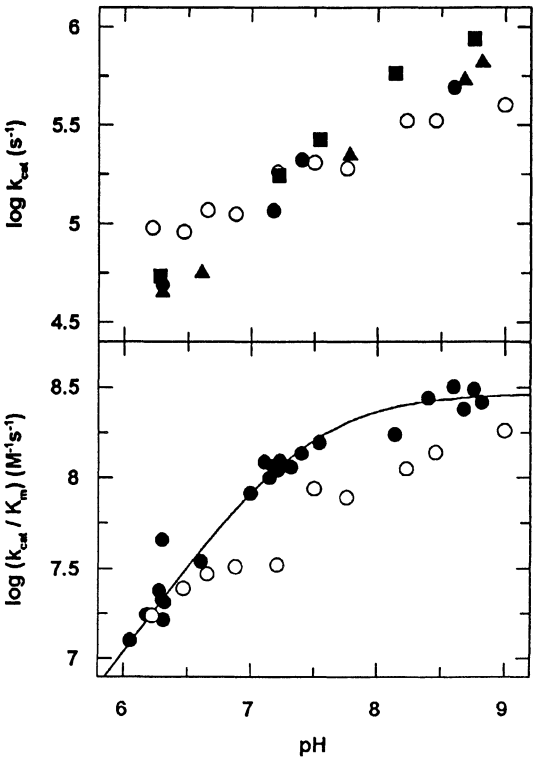

Fig. 5 pH dependence of the parameters k_{cat} and k_{cat}/K_m for the CO_2 hydration catalysed by the H208A mutant and unmutated pea CA. Filled symbols represent the mutant and the open circles represents the unmutated enzyme. The values refer to the activity per subunit.

Assuming that a similar model for the catalytic mechanism is valid for pea CA as for HCA II, the pH dependence of k_{cat}/K_m will reflect the titration of the zinc-bound water molecule. In the mutant H208A a pK_a value of 7.4 is found while the titration of the unmutated enzyme is complex and no pK_a value can be evaluated,

but it appears that the zinc-bound water molecule is not fully ionised at pH 9. Anions bind stronger to the protonated enzyme form, thus, the weaker binding of SCN^- to the H208A mutant at high pH agree with the changed titration of the zinc-bound water molecule. The large value of the buffer K_m found for the H208A mutant at low pH makes it tempting to speculate that His208 at this pH is somehow involved in the proton-transfer step of the catalysed reaction. On the other hand, it might be that the H208A mutation causes a local conformational change affecting the accessibility of the buffer molecule to the proton-donating group of the enzyme. In addition the presence of His 208 makes the enzyme less sensitive to oxidation, thus, stabilizing the reduced and active state of pea CA.

FUTURE PROSPECTS

6. Understanding the pea CA enzymatic mechanism

6.1 X-ray structure

Knowledge of the three-dimensional structure of an enzyme is invaluable for the correct interpretation of kinetic results, especially when site-directed mutants are investigated. We are collaborating with crystallographers in Uppsala with the aim to solve the X-ray structure of pea CA. However, well-diffracting crystals have not been obtained so far. One possible explanation is the requirement of a reduced environment for enzyme activity. If some of the oxygen-sensitive cysteine residues are reduced while some of them are in the oxidised state, the crystals formed will probable be disordered. A possible solution might be to block free cysteines in the enzyme or to produce a mutant lacking the cysteine residues that confer the oxidation sensitivity (see 6.3 p 34). Another approach would be to change the source of the enzyme.

6.2 Kinetic studies

So far all presented kinetic data on plant CAs have been obtained under steady-state conditions. Measurements at chemical equilibrium can give important additional information. For CAs from the animal group two different methods have been used to follow the interconversion between substrate and product at equilibrium. With ^{13}C NMR the CO_2 - HCO_3^- exchange rates have been estimated from line broadening (Simonsson et al., 1979). In addition, mass spectrometry using ^{18}O-labelled substrates has been used (Silverman et al., 1979). From results of such measurements with pea CA it might be possible to determine if the partially rate-

determining proton transfer step and the buffer step are within or outside the CO_2 - HCO_3^- interconversion sequence.

6.3 SH groups

To clarify the function of the cysteine residues in pea CA the conserved cysteines 159 and 222 should also be studied. Furthermore, the mutants at positions 269 and 272 have to be obtained in a soluble form (see 5.4 p 29). One way to obtain soluble mutants might be to denature and refold the material in the inclusion bodies obtained in *E. coli*. However, it might be difficult to interpret the results of such an approach since we do not now how active the mutants are. A low activity could tell us that the replaced residue is needed for enzyme activity but could equally well tell us that we have not been able to refold the enzyme completely. Another approach is to overexpress chaperones together with the pea CA mutants. One could either use the *E. coli* chaperones GroEL and GroES or, if this does not work, the chloroplastic chaperonins cpn10 and cpn60.

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