The Sulfhydryl Groups of Cys 269 and Cys 272 Are Critical for the Oligomeric State of Chloroplast Carbonic Anhydrase from *Pisum sativum*†

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ABSTRACT: Chloroplast carbonic anhydrase is dependent on a reducing environment to retain its catalytic activity. To investigate the properties of the three accessible cysteine residues of pea carbonic anhydrase, mutants were made in which Ala or Ser substituted for C165, C269, and C272. The mutants at position 165 were found to be spectroscopically similarly to the wild-type. They have a high catalytic activity, and are also sensitive to oxidation. In contrast, both C269 and C272 were found to be critical both for the structure and for the catalytic activity. All mutants with substitutions at either of these two positions had to be co-overexpressed with GroES/EL chaperones to give soluble enzyme in *Escherichia coli*. The k_{cat} values were decreased by 2 and 3 orders of magnitude for the C272A and C269A mutants, respectively, and the K_m values were increased approximately 7 times. However, the binding of the inhibitor ethoxyzolamide was only slightly weakened. The near-UV CD spectra were found to be changed in both sign and intensity compared to that of the wild-type, and the far-UV spectra indicate some loss of α-helix structure. Moreover, the quaternary structure was changed from the wild-type octameric to tetrameric in these mutants. The results indicate that mutation of either of these cysteines causes minor structural changes around at least one of the two tryptophans of the subunit. Furthermore, the data demonstrate that C269 and C272 are involved in the interaction between subunits and are necessary for a proper structure at the tetramer−tetramer interface.

Carbonic anhydrase (CA; EC 4.2.1.1)[1] is a zinc-containing enzyme which catalyzes the reversible hydration of CO_2. It is a ubiquitous enzyme that has been found in animals, photosynthesizing organisms, and some nonphotosynthetic prokaryotes. From sequence homologies, the CAs are found to belong to three genetically distinct families, designated α-, β-, and γ-CA (Hewett-Emmett & Tashian, 1996). All known animal CAs, together with two CAs of different locations in the unicellular green alga *Chlamydomonas reinhardtii* (Karlsson et al., 1995; Fukuzawa et al., 1990), and a prokaryotic CA from *Neisseria gonorrhoaea* (Hewett-Emmett & Tashian, 1996) belong to the α-family. The most extensively studied CAs belong to this family. β-CAs have been shown to be present in both C3 and C4 monocotyledonous and dicotyledonous plants. They have also been found in certain eubacteria such as *Haemophilus influenzae* (Casari et al., 1994) and *Escherichia coli* as part of the *cyn* operon (Guilloton et al., 1992), and in the cyanobacterium *Synechococcus* (Fukuzawa et al., 1992). Only one enzyme has been identified and characterized from the most recently identified gene family, γ-CA, from the archaebacterium *Methanosarcina thermophila* (Alber & Ferry, 1994).

The crystal structures have been solved for several of the mammalian α-CA forms (Eriksson & Liljas, 1993; Boriack-Sjodin et al., 1995; Kannan et al., 1975; Eriksson et al., 1988). The overall folds of these monomeric isozymes are highly similar with a 10-stranded, mainly antiparallel, β-sheet as the dominating secondary structure. The catalytically active zinc is ligated to three histidine residues with a water molecule as a fourth ligand, giving almost tetrahedral coordination geometry. The recently-published crystal structure of the trimeric γ-CA from *M. thermophila* was found to be entirely different, with the active site located between the subunits of the enzyme (Kisker et al., 1996). The main secondary structures are several parallel β-sheets forming a left-handed β-helix. Despite these structural differences, the binding of the zinc is very similar to its binding in α-CAs. Information concerning the structure of the β-CAs is more limited, and no three-dimensional structure is yet known for any enzyme belonging to this family. Alignment of all β-CAs so far known shows invariant amino acid residues at around 20 positions, of which most are found within 2 regions. Extended X-ray absorption fine structure (EXAFS) analysis of spinach CA suggests a Cys-His-Cys-H_2O ligand scheme to bind the zinc ion (Bracey et al., 1994; Rowlett et al., 1994). Within the first conserved region, we find one invariant Cys, and the other region includes a His and a Cys that are conserved and are likely to constitute the zinc ligands. According to primary structures, the β-CAs can be divided into three groups represented by dicotyledons, monocotyledons, and prokaryotes with a high degree of sequence homology within each group. The three groups of β-CAs differ in their quaternary structures. The native molecular

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[1] Abbreviations: CA, carbonic anhydrase; CD, circular dichroism; EXAFS, extended X-ray absorption fine structure; DMS, dimethyl suberimidate dihydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GroELS, *gro*EL and *gro*ES gene products; MOPS, 4-morpholinepropanesulfonic acid; TAPS, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino]-1-propanesulfonic acid; TCEP, tris(2-carboxyethyl)-phosphine.

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mass of the enzyme from C3 dicotyledonous plants has been reported to vary between 140 and 250 kDa, with a subunit mass of 24−34 kDa (Reed & Graham, 1981). The oligomeric state is therefore assumed to be a hexamer or an octamer of identical subunits (Rumeau et al., 1996; Johansson & Forsman, 1993; Reed & Graham, 1981), but the exact number of subunits has not been unambiguously shown. There is no evidence for a covalent linkage between the subunits. CA from monocotyledonous plants has been suggested to be a dimer (Atkins, 1974; Atkins et al., 1972), and the prokaryotic CA from *E. coli* was also reported to be an oligomer, most likely a tetramer or a dimer depending on experimental conditions (Guilloton et al., 1992).

Most biochemical studies on β-CAs have been done on CAs from C3 dicotyledons. In these plants, the enzyme is mainly located in the chloroplast stroma, directed to the plastid by an N-terminal chloroplast transit peptide (Forsman & Pilon, 1995). Kinetic studies have been done on CA from pea (Johansson & Forsman, 1993, 1994) and spinach (Rowlett et al., 1994). Both have high catalytic efficiency with k_{cat} values between 10^5 and 10^6 s^{-1} and k_{cat}/K_m values of 10^7-10^8 M^{-1} s^{-1} at high pH. The kinetic data are consistent with the general mechanism proposed for the high-activity α-CA isozymes (Silverman & Lindskog, 1988). This maintains that the zinc-bound water molecule is ionized to a hydroxide ion, which is the catalytically active group. The β-CAs are also found to be sensitive to the classical CA inhibitors: sulfonamides and monovalent anions (Reed & Graham, 1981). Most dicotyledonous plant CAs that have been purified and characterized are reported to be dependent on a reducing agent to retain catalytic activity (Tobin, 1970; Rumeau et al., 1996; Cybulsky et al., 1979; Johansson & Forsman, 1993). For pea CA, we found that the oxidized, inactive enzyme could be reactivated by the addition of a reducing agent. However, only 60% of the activity was regained, at best, suggesting an irreversible alteration of the protein. The β-CAs from the two prokaryotes *E. coli* and *Synechococcus* do not seem to be sensitive to oxidation (Guilloton et al., 1992; Price et al., 1992). In fact, the activity of the *Synechococcus* enzyme was reduced by 80% when incubated for 45 min in the presence of 0.1−10 mM dithiothreitol.

In this study, we have focused on the importance of the cysteine residues of the chloroplastic CA from pea. Each subunit contains five cysteines. Two of these are conserved among all known β-CAs and are likely to be zinc ligands. They are at positions 159 and 222 in the pea CA sequence, starting from the initial methionine of the transit peptide, and are not included in this study. The other three cysteines, at positions 165, 269, and 272, should include the one(s) responsible for the oxidation sensitivity. C165 is found in all higher plant CAs, in the immediate vicinity of the conserved region which includes C159. The remaining two Cys residues are closely spaced at positions 269 and 272. C269 is conserved in CA from both mono- and di-cotyledons, while C272 is present in all sequenced dicotyledonous CAs but is absent from all monocotyledonous CAs. In the bacterial β-CAs, which are independent of reducing agents, the corresponding positions are occupied by residues other than cysteines. Our initial aim was to use site-directed mutagenesis in order to make an enzyme variant that is insensitive to oxidation. We have changed the three cysteines to alanines and serines. The resulting mutants show that C165 does not mediate the dependence on a reducing agent, and this residue is not necessary for catalytic activity. In contrast, the mutants with substitutions at positions C269 or C272 have drastically reduced catalytic activities. The cysteines at these two positions are also found to be of vital importance for maintaining the quaternary structure. Thus, we have not been able to make a mutant with a high catalytic activity that is insensitive to oxidation. Instead we report the importance of the cysteine −SH groups for preventing irreversible structural changes that inactivate the enzyme.

EXPERIMENTAL PROCEDURES

Plasmids. The mutagenesis/expression plasmid pPCAs, encoding wild-type pea CA under control of the T7 RNA polymerase promoter, has been previously described (Johansson & Forsman, 1992). This plasmid also contains an F1 origin of replication and an ampicillin resistance gene. PCR was used to amplify the GroES and GroEL genes from *E. coli* DNA and at the same time introduce a Shine−Dalgarno sequence upstream of the start codon. The resulting product was ligated into the PCR-cloning vector pCR™II (Invitrogen) downstream from a T7 promoter, and upstream from the unique *Hin*dIII site. A second *Hin*dIII site was introduced upstream from the T7 promoter. Cleavage with *Hin*dIII generated a fragment containing the GroES and GroEL genes under control of the T7 promoter together with the kanamycin resistance gene of the plasmid. Plasmid p15AGroELS was obtained by cloning this fragment into the unique *Hin*dIII site of plasmid pACYC184 (Rose, 1988). pACYC184 has a p15A replicon making it compatible with plasmids having a ColE1-type replicon like pPCAs. p15AGroELS also carries the chloramphenicol acetyltransferase gene.

Mutagenesis. In vitro site-directed mutagenesis was performed essentially according to the method of Kunkel (1985), and mutants were identified by direct sequencing of the plasmid DNA. Plasmid containing the mutation was used to transform *E. coli* BL21(DE3) (Studier & Moffatt, 1986) which carries a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. To generate bacteria co-overexpressing GroELS and pea CA, *E. coli* BL21(DE3) harboring p15AGroELS was transformed with a pPCAs mutant plasmid. The complete pea CA coding region was checked by plasmid sequencing before the clone was used for protein production. All sequencing was performed according to the chain termination method (Sanger et al., 1977).

The following mutants were constructed for this study: C165A, C165S, C269A, C272A, and C269S/C272S.

Protein Production and Purification. E. coli BL21(DE3) harboring wild-type, C165A, or C165S mutant plasmids were grown at 23 °C in rich medium complemented with 100 mg/L ampicillin to $A_{660} = 0.5$, at which point pea CA synthesis was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested after an additional 15 h and resuspended in 50 mM Tris−H_2SO_4 (pH 8.0) before mechanical disruption using a Bead-Beater (BioSpec Products). The enzyme was purified by affinity chromatography using *p*-(aminomethyl)benzenesulfonamide hydrochloride (Sigma) coupled to epoxy-activated Sepharose 6B (Pharmacia Biotech) as previously described (Johansson & Forsman, 1993). The protein was eluted with 0.1 M NaN_3

followed by dialysis against several changes of degassed 10 mM Bistris−H_2SO_4 (pH 6.8) with or without the addition of 10−30 mM cysteine or 0.1 mM tris(2-carboxyethyl)-phosphine (TCEP; Pierce) as a reducing agent.

The mutants C269A, C272A, and C269S/C272S were produced together with overexpression of the GroELS chaperones. The growth conditions were as above, and the medium was supplemented with 100 mg/L ampicillin and 25 mg/L each of kanamycin and chloramphenicol. Enzyme purification was as for the wild-type, but elution from the affinity column was at 0.8 M NaN_3.

The purity of the preparations was verified using SDS−PAGE and Coomassie Brilliant Blue staining. The enzymes were stored in small aliquots under N_2(g) at −20 °C. Protein concentrations were determined either by using the Coomassie Plus Protein Assay Reagent (Pierce) and bovine serum albumin as standard or from the absorbance at 280 nm. The molar extinction coefficients were calculated as described by Gill and von Hippel (1989). A value of 28 200 M^{-1} cm^{-1} was obtained for the wild-type, C165A, and C165S enzymes and 24 700 M^{-1} cm^{-1} for the other mutants. The enzyme concentrations refer to the amount of subunits based on a molecular mass of 24.2 kDa.

Size-Exclusion Chromatography. Apparent molecular masses of the native enzymes were estimated by gel filtration on a Superose 12 HR 10/30 column (Pharmacia Biotech) using 20 mM Tris−H_2SO_4, 0.1 M Na_2SO_4 (pH 7.5) as eluant. The column was calibrated with a gel filtration standard (Bio-Rad) containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

Chemical Cross-Linking. Dimethyl suberimidate dihydrochloride (DMS; Pierce) was prepared at a concentration of 0.25 or 0.5 M in 50 mM sodium borate, 50% dimethyl sulfoxide with the addition of 1 M NaOH to adjust the final pH to 8.5. The enzyme was dialyzed against 50 mM sodium borate buffer (pH 8.5) and mixed with different concentrations of DMS at a final enzyme concentration of 25 μM. The reaction was allowed to proceed for 1 h at 23 °C, at which point an excess of sodium glycine (pH 8.5) was added to quench the reaction. Samples containing 5 μg of enzyme were prepared for SDS−gel electrophoresis.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were obtained at 23 °C using a Jasco-720 spectropolarimeter. Each spectrum was the result of three scans using a band width of 2 nm. The samples contained 10 mM potassium phosphate buffer at pH 7.3, and the far-UV spectra were recorded using a 1 mm quartz cell and 0.35 mg/mL protein. Near-UV spectra were recorded using a 4 mm quartz cell and 1 mg/mL protein. The samples were dialyzed against the phosphate buffer, and the protein concentrations were determined after dialysis and centrifugation by measuring the absorbance at 280 nm. Spectra recorded below 200 nm followed by the same protocol except that a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France) was used, the sample concentration was 0.5 mg/mL, and a 0.5 mm quartz cell was used. The observed ellipticities were converted to mean residue ellipticity, [Θ], by using the equation:

$$[\Theta] = \Theta_{obs}(\mathrm{mrw})/10cl$$

where Θ_{obs} is the observed ellipticity in degrees, mrw is the mean residue molecular weight based on a molecular mass of 24.2 kDa and 222 amino acids, c is the protein concentration in grams per milliliter, and l is the path length of the cell in centimeters.

Protein Fluorescence and Binding of the Sulfonamide Ethoxyzolamide. Ethoxyzolamide (6-ethoxy-2-benzothiazolesulfonamide) was dissolved in 95% ethanol to a concentration of 1 mM. Its molar extinction coefficient was determined to be 7.5×10^3 M^{-1} cm^{-1} at 280 nm and 1×10^3 M^{-1} cm^{-1} at 332 nm. Binding of the inhibitor to pea CA was observed to cause quenching of the protein fluorescence. Wild-type or mutant enzyme at a concentration of 2×10^{-7} M in 50 mM Bistris−H_2SO_4 (pH 6.6) was excited at 280 nm, and the emission was recorded at 332 nm using a Shimadzu RF-5000 spectrofluorophotometer and an effective path length of 2 mm. Titration with ethoxyzolamide was done by repeated additions of small aliquots of 1 mM ethoxyzolamide stock solution. All measurements were taken at 23 °C after an incubation time of 2 min. No time-dependent change in the signal was observed when the incubation time was increased, and so we assume to have reached equilibrium conditions. The fluorescence data were corrected for background fluorescence, for dilution of the sample, and for inner filter effects caused by ethoxyzolamide absorption at 280 nm. The corrected fluorescence intensities were used to calculate the dissociation constant of the inhibitor, K_i, by fitting the data to the equation:

$$\Delta F = \Delta F_{max}[\mathrm{I}]/(K_i + [\mathrm{I}])$$

where ΔF is the difference in fluorescence intensity of protein in the absence and presence of ethoxyzolamide, and [I] is the free inhibitor concentration. Nonlinear data fitting was done using the GraFit program (Erithacus Software Ltd., England).

Intrinsic tryptophan fluorescence was recorded at 23 °C by registering the emission between 310 and 370 nm using a band width of 10 nm after excitation at 295 nm with a band width of 3 nm. The enzyme concentrations were 2.3 μM in 50 mM Bistris−H_2SO_4 (pH 6.6).

Kinetic Measurements. Kinetic parameters were measured in an Applied Photophysics DX-17MV sequential stopped-flow spectrofluorometer, or a Hi-Tech stopped-flow spectrophotometer, at 25°C by the changing pH-indicator method (Khalifah, 1971). The buffer/indicator pairs used were TAPS with *m*-cresol purple, measured at 578 nm, and MOPS with 4-nitrophenol measured at 400 nm. All buffers contained 10 μM EDTA and 1 mM dithiothreitol to prevent oxidation. The enzyme/buffer solutions were stored in a gas-tight syringe until use. The initial rates were calculated by fitting the first part of the curve to a first-order rate equation. Initial rate data were fitted by nonlinear regression to the Michaelis−Menten equation using the GraFit program (Erithacus Software Ltd., England).

Modification of Pea CA with the Sulfhydryl Reagent DTNB. Enzyme purified in the presence of a reducing agent was dialyzed overnight at +4 °C against degassed 0.1 M potassium phosphate, 1 mM EDTA, pH 7.3, under N_2(g). Modification of −SH groups was carried out at 25 °C in a cuvette containing the same buffer, 6 μM enzyme and 150 μM 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which was placed in the chamber of a spectrophotometer. In the blank reaction, the dialysis fluid was substituted for pea CA

Table 1: Isolation of Pea CA Mutants and CO_2-Hydration Activity Measured at 25 °C in 50 mM TAPS Buffer (pH 9.0)

enzyme	GroELS	yield[a] (mg)	v/[E][b] (s^{-1})	k_{cat} (ms^{-1})	K_m (mM)
wild-type	−	150	240000	400 ± 12	2.2 ± 0.2
C165A	−	40	180000	*c*	*c*
C165S	−	90	160000	*c*	*c*
C269A	+	115	50	0.15 ± 0.07	16 ± 12
C272A	+	38	250	1.3 ± 0.1	16 ± 2
C269S/C272S	+	76	0		

[a] Amount of isolated enzyme obtained from 2.5 L of cell culture. [b] Specific activity for the isolated enzyme at 17 mM CO_2. [c] Non-Michaelis−Menten kinetics.

solution, and the difference in absorbance at 412 nm between the sample and the blank was measured. The number of modified −SH groups was calculated using a molar extinction coefficient of 14 150 (Riddles et al., 1983). When the reaction was performed in the presence of ethoxyzolamide, a 20 μM sample of the inhibitor was added to the enzyme-containing reaction prior to the addition of DTNB. The enzyme was oxidized using 1 mM diamide (Serva) in 10 mM sodium phosphate (pH 7.3) at room temperature. Oxidized enzyme was separated from low molecular weight compounds on a Sephadex G-25 column (NAP 10, Pharmacia Biotech).

RESULTS

The mutants fall into two main categories: the C165A and C165S mutants (designated the C165 mutants) and the single and double mutants involving substitutions at positions 269 and 272, designated the C269−C272 mutants.

Enzyme Isolation. The isolation of the different mutants is summarized in Table 1. Wild-type enzyme and the C165 mutants could be produced in a soluble form in *E. coli*. The C269−C272 mutants had a similar expression level, but accumulated as inclusion bodies and could not be extracted from the cells in a soluble form. This problem was solved by co-overexpression with the bacterial chaperones GroELS. A high level of chaperones inside the cells seems to prevent misfolding and aggregation of these mutant forms, and a high yield of soluble enzyme was obtained. The amount of purified enzyme varied depending on the mutant (Table 1), from 23% of the wild-type yield, for the C272A mutant, to 77% of wild-type for the C269A mutant.

All mutants in this study bound to the sulfonamide affinity chromatography column. The wild-type and the C165 mutants were eluted at 0.1 M NaN_3, while the azide concentration had to be increased to 0.8 M to elute the C269−C272 mutants. Only the wild-type and C165 mutants possess highly specific CO_2-hydration activity, expressed as v/[E] (Table 1). All other mutants have extremely low activities, at least 3 orders of magnitude lower than wild-type, or are completely inactive. There is some uncertainty concerning the exact figures, since the uncatalyzed reaction constitutes a major part of the measured activity. Nevertheless, the activities obtained for the C269A and C272A mutants clearly differ significantly from the autocatalyzed reaction.

Enzyme Kinetics and Inhibition. The C165S and C165A mutants have similar CO_2-hydration activities, of the same order as the wild-type enzyme. However, strong deviations from Michaelis−Menten kinetics were obtained at both high and neutral pH. Eadie−Hofstee plots of the data obtained at pH 8.6 in 50 mM TAPS buffer are presented in Figure 1. Because of their curvature, we have not determined any kinetic parameters. To facilitate comparison, a line representing the fitted data for the wild-type enzyme in the same buffer (pH 8.5) has been added to the figure. The C165 mutants, like the wild-type, are inactivated upon oxidation and can be reactivated by the addition of a reducing agent. Thus, the Cys at position 165 is not directly involved in the catalytic event, and it is not responsible for the oxidation sensitivity. Of the C269−C272 mutants, kinetic parameters were only determined for C269A and C272A (Table 1). The k_{cat} values of the C272A and C269A mutants are 2 and 3 orders of magnitude lower than that of the wild-type, respectively, and the K_m values are approximately 7 times higher. When these mutants were purified without addition of a reducing agent to the dialysis buffer, the activity of the C269A mutant was not significantly changed while the activity of the C272A mutant was reduced by 66%.

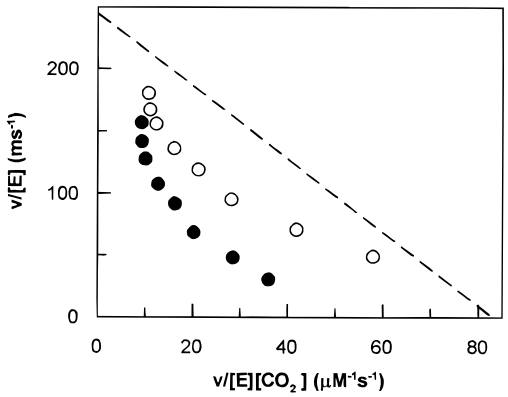

FIGURE 1: Eadie−Hofstee plots of the initial rates of CO_2 hydration catalyzed by the C165 mutants in 50 mM TAPS (pH 8.6) and 25 °C. Enzyme concentrations were 0.07 mM for C165A and 0.14 μM for C165S. Na_2SO_4 was added to a total ionic strength of 50 mM. (○) C165A; (●) C165S. The dashed line represents fitted data for the wild-type enzyme under the same conditions [data from Johansson and Forsman (1993)].

The observation that the C269−C272 mutants did not elute from the affinity resin until the azide concentration was increased 8 times indicates that the mutations change the affinities of the enzyme for the inhibitors. We therefore determined the sulfonamide dissociation constants. Due to the extremely low catalytic activities of these mutants, the strength of binding could not be estimated from inhibition of the CO_2 hydration. The fluorescent sulfonamide dansylamide has frequently been used for determining K_i values of mutants of human CA, but does not bind strongly enough to pea CA for this purpose. Instead we utilized the quenching of the intrinsic protein fluorescence observed upon sulfonamide binding. Ethoxyzolamide had previously been found to be a very strong inhibitor of the pea CA activity (Johansson & Forsman, 1993) and was used here to measure the strength of binding to the mutants. The results are presented in Table 2, and it is evident that all these mutants bind ethoxyzolamide more weakly than the wild-type does, their K_i values being between 5 and 10 times higher. Nevertheless, the derived dissociation constants of 10−20 μM show that the interactions are reasonably strong. Further, from the behavior of the mutants on the sulfonamide affinity column, we conclude that the affinity for azide, and possibly other anions, must also be lowered.

Table 2: Binding of Ethoxyzolamide to Pea CA[a]

enzyme	K_i (μM)
wild-type	1.8 ± 0.3
C269A	11 ± 2
C272A	13 ± 2
C269S/C272S	19 ± 2

[a] Calculated from protein fluorescence quenching at 23 °C in 50 mM Bistris−H_2SO_4 (pH 6.6).

Reaction with DTNB. Free thiol groups were labeled with DTNB to determine the accessibility of Cys residues in different enzyme variants. Using TCEP-reduced enzymes, 2.9 thiols/subunit and 1.9 thiols/subunit can react in the wild-type and the C165A mutant, respectively. This result fits well with the expected values, considering that of the five cysteines present in each subunit of the wild-type, two are likely to be coordinated to the zinc and are thus unlikely to be able to react with DTNB. Cybulsky et al. (1979) noted, for the spinach CA, that the presence of the inhibitor, acetazolamide, reduced the amount of 2-nitro-5-thiobenzoate (TNB) anion released, and increased the half time of the reaction. When we add ethoxyzolamide to pea CA before addition of DTNB, we still find 3.1 −SH groups react, and a marginal effect on the half time of the reaction, which increases from 2.4 min to 5 min. Therefore, it does not seem likely that any of the accessible cysteine residues could be located in the close vicinity of the zinc ion. Having obtained this result, we expected the C269A and C272A mutants to react with 2 equiv of DTNB each, and the C269S/C272S double mutant to react with 1 equiv. To our surprise, when following the reaction for a time of 6 h, we found these three mutants react with only 0.2, 0.3, and 0.1 equiv of DTNB, respectively. In other words, the remaining cysteines, C165 and C269 or C272, are no longer accessible, which means that mutations at either position 269 or position 272 must induce a similar conformational change. The possibility that a disulfide bridge has formed between C165 and C269 or C272 does not seem likely since the double mutant gave the same result as the single mutants, and also because TCEP-reduced enzyme was used. SDS−PAGE in the absence of reducing agents gave no evidence of the formation of disulfide bridges either (data not shown).

Oxidation of the wild-type and C165A mutant by diamide for 60 min gives complete loss of the catalytic activity with a half time of 6.6 min. This oxidation is irreversible; no activity at all can be regained by the addition of 5 mM TCEP or 100 mM 2-mercaptoethanol. Theoretically, if a disulfide bridge has formed upon oxidation, the wild-type can react with 1 equiv of DTNB while the C165A mutant will not have any accessible −SH groups. We find, however, that DTNB is unable to react with any Cys residue in either of the two oxidized enzymes. Since the Cys at position 165 does not react with DTNB in the oxidized wild-type enzyme, the oxidation must induce a structural change that makes this residue inaccessible. The disulfide bond formed by treatment with diamide must be present on a single polypeptide, since the electrophoretic mobilities are the same for reduced and oxidized enzymes under both nondenaturing and denaturing conditions, and in the presence or absence of reducing agents in the sample buffer.

Determination of the Oligomeric Structure of Pea CA. The native molecular masses were estimated and found to differ between the C165 mutants and the C269−C272 mutants. The nondenaturing gel shown in Figure 2 clearly shows that the mobilities of the C269−C272 mutants (lanes 2, 4, and 5) are very much higher than those of C165A and the wild-type protein (lanes 1 and 3), which correspond to molecular masses of 100 and 220 kDa, respectively. No protein of lower molecular mass was detected in any case. This finding was confirmed by size-exclusion chromatography. Using the Bio-Rad gel filtration standards, we obtain apparent native molecular mass values of around 180 kDa for both the wild-type and the C165A mutant. Both of these enzymes elute as single, sharp peaks. The C269−C272 mutants all elute with a major peak corresponding to approximately 70 kDa. For the C269A mutant, trace amounts of protein with higher apparent molecular mass values is found. Thus, in all mutants in which we have replaced Cys 269 or Cys 272 with either an Ala or a Ser, the native sizes seem to be reduced to half of that of the wild-type. This means that the oligomeric structure must have changed from an octamer or a hexamer to a tetramer or a trimer.

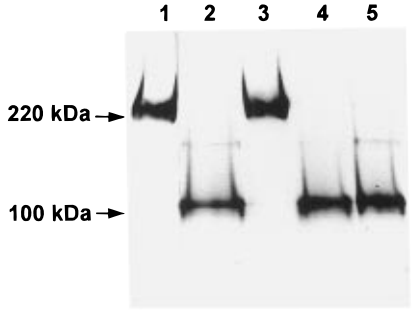

FIGURE 2: Estimation of the apparent molecular mass of wild-type and mutant pea CA. Analysis on a 5−11% nondenaturing polyacrylamide gel. Lane 1, C165A; lane 2, C269S/C272S; lane 3, wild-type; lane 4, C272A; lane 5, C269A.

This finding encouraged us to use chemical cross-linking to determine the number of subunits that form one oligomer of the mutant. Cross-linking of the wild-type enzyme has not been successful in our hands using agents that both react with primary amines and have spacer-arm lengths ranging from 8.6 to 16.1 Å. The problem was difficulty in obtaining a full ladder on the SDS−gel with a clearly visible band for each *n*-mer, ranging from monomer up to hexa- or octamer. Monomers and dimers can be obtained, but for the larger products, several very faint bands appear, resulting in a smear. Increasing the cross-linker concentration gives some material of high molecular mass that fails to enter the gel, and also a distinct band of molecular mass above 200 kDa that probably represents the fully cross-linked oligomer (data not shown). This result was not clear enough to tell us unambiguously whether the enzyme is an octamer or a hexamer. However, the same experiment with the C269A mutant gave much more clear-cut results. DMS, which reacts with primary amines and has a spacer arm of 11 Å, was the most suitable cross-linker tested, and it was used at concentrations up to 200 mM. The amount of cross-linked species produced increases up to a concentration of 37 mM DMS, but further increases in DMS concentration give no higher yield either of cross-linked species or of products of higher molecular mass. The result, using 100 and 200 mM DMS, is shown in Figure 3 and the three distinct bands obtained have apparent molecular masses of 25, 50, and 100 kDa.

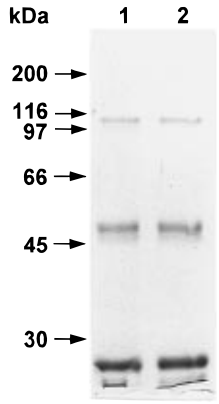

FIGURE 3: SDS−PAGE analysis of cross-linked C269A. 25 μM enzyme was used for cross-linking in 50 mM borate buffer (pH 8.5) and analyzed on an 11% SDS−gel. DMS concentrations of 100 mM (lane 1) and 200 mM (lane 2) are shown. Molecular- size markers (kDa) are to the left.

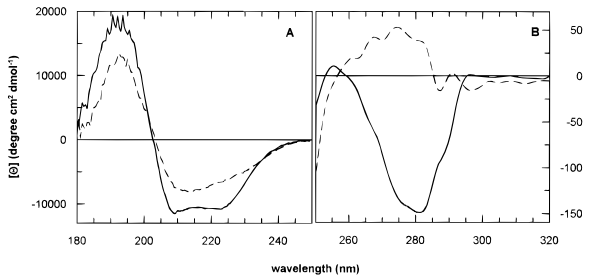

FIGURE 4: CD spectra of wild-type pea CA and the C269A mutant. (A) Far-UV region; (B) near-UV region. (—) wild-type; (- - -) C269A.

We conclude that these bands represent monomers, dimers, and tetramers, and that the symmetry of the tetramer is such that the probability of trimer formation is low. Moreover, since this shows that the C269A mutant is a tetramer, the wild-type pea CA must be an octamer.

Spectral Properties. To investigate whether the formation of a tetramer instead of an octamer results in changes in the secondary and tertiary structures, we recorded the CD spectra of the wild-type, and the C165A, C269A and C269S/C272S mutants. Since the investigations presented in this study have shown that the C165 mutants have similar characteristics to the wild-type, it could be expected that the overall structures of these mutants are essentially unchanged. Indeed, the CD spectra for the C165A mutant overlap with those of the wild-type over the entire wavelength region. We also find that the spectra for the C269S/C272S mutant are highly similar to those recorded for the C269A mutant. For the wild-type and C269A enzymes, the measurements were extended down to 180 nm to get a more significant estimate of the secondary structure, and the spectra are presented in Figure 4. If we first consider the far-UV region (Figure 4A), the wild-type spectrum has two intense negative bands at about 223 nm ($[\theta] = -10\,810$) and 209 nm ($[\theta] = -11\,510$), and an intense positive band at 192 nm ($[\theta] = 19\,278$). This strongly indicates that the α-helix is the dominating secondary structure in wild-type pea CA (Johnson, 1990). The CD spectrum of the tetrameric C269A mutant is much less intense, with molar ellipticities of −6582, −7424, and 12835 at 223 nm, 209 nm, and 192 nm, respectively. Qualitatively, this could mean that there is a loss of α-helix in the transition from the octamer to the tetramer. In the near-UV region (Figure 4B), a more drastic change is observed both in the sign and in the magnitude of the bands. This region reflects the surrounding of the aromatic residues, and thus the immediate structural and electronic environment of the chromophores must have changed. This might reflect subtle changes in the tertiary structure due to the changed quaternary structure. To further investigate the spectral properties of the tryptophans, which give the major contribution to the near-UV CD spectrum, the intrinsic protein fluorescence was measured for the wild-type and the C269−C272 mutants. Excitation was at 295 nm, where only the tryptophans are optically active, and the emission spectra for the wild-type enzyme, the C269A single mutant, and the C269S/C272S double mutant are shown in Figure 5. The spectrum for the C272A mutant does not differ from those of these two mutants. We see that the fluorescence maxima for the mutants are at the same wavelength as for the unmutated enzyme (around 335 nm), while the fluorescence intensities of all mutants are increased by at least 30%. Since the mutant spectra have not shifted either to the red or to the blue, the surroundings of the tryptophans must be equally hydrophobic as in the wild-type. This indicates that the tryptophans are not located directly at a subunit surface that becomes exposed to the solvent when the octamer is split into a tetramer. However, the increase in fluorescence intensities for the mutants suggests that the possibilities of energy transfer between tryptophans or to a quencher in these mutants are different from those of the wild-type enzyme.

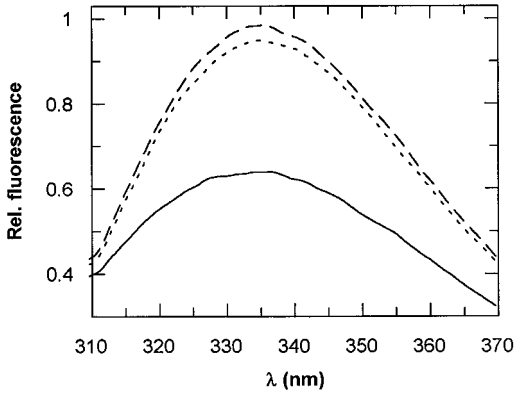

FIGURE 5: Fluorescence emission spectra of wild-type pea CA (—), C269A (— —) and C269S/C272S (- - -) after excitation at 295 nm.

DISCUSSION

This study has focused on the importance of the cysteine sulfhydryl groups of higher-plant CA, excluding the two Cys presumed to constitute the zinc ligands (Rowlett et al., 1994; Bracey et al., 1994). Of the remaining cysteines, we find that C165, present in both mono- and dicotyledonous plant CAs, is not directly involved in the catalytic event and the C165 mutants have very similar spectroscopic characteristics to the wild-type enzyme. However, the deviation from Michaelis−Menten kinetics indicates that C165 could be indirectly involved in the catalysis. Moreover, structural changes induced by the C269−C272 mutants also involve position 165, since in these mutants C165 is no longer accessible to reaction with DTNB. The cysteines at positions 269 and 272 are found to be critical both for the structure and for the catalytic activity. The most striking change is the altered oligomeric state. We have shown the wild-type enzyme to be an octamer, but replacing either or both C269 and C272 results in formation of tetramers. The importance

of the cysteines at positions 269 and 272 for functional conformation also seems to involve the folding process. Co-overexpression of the chaperones GroEL and GroES was necessary to avoid aggregation and precipitation of misfolded protein, and it is possible that the folding of the subunits and the formation of the oligomers are two interdependent processes.

A model of the quaternary structure of chick pea leaf CA, based on electron microscopy of the molecule, has been presented by Aliev et al. (1986). They suggested a *P 422* symmetry for the eight subunits. The authors found indications of a double-layered structure of the molecule, each layer being a tetramer, which fits well with our results from the cysteine mutants. Cross-linking of the C269A mutant do not give formation of any trimers, which indicates that one of the subunit contact sides is in more intimate contact than the other, and thus reacts more readily with the cross-linker, resulting in dimer formation. Cross-linking across the other interface then gives the tetramer. This indicates that the hypothesis by Miller (1989) that proteins composed of 2*n* identical subunits are generally assembled as *n*-mers of dimers might also be valid for pea CA.

Computation of the secondary structure of the pea CA sequence by the methods of Rost and Sander (1994) and Garnier et al. (1978) predicts the region between residues 265 and 281 to be an α-helix. Helical wheel analysis shows that C269 and C272 would become neighbors along the helix and part of a hydrophobic helical face, while the opposing side is dominated by hydrophilic and charged residues. We speculate that this helix constitutes an important component of the tetramer−tetramer interface, necessary for a strong interaction between the subunits. Indeed, the less intense far-UV CD spectra for the tetrameric C269−C272 mutants indicate that there is a loss of α-helix structure. A similar change of the CD spectrum has been observed for the hexameric $12S_H$ subunit of transcarboxylase upon dissociation into monomers (Hennessey et al., 1982). The cysteine side chains cannot be buried in the center of the contact surface since in the wild-type they are both accessible to reaction with DTNB. Replacing the cysteines with either a hydrophobic (Ala) or a hydrophilic (Ser) residue results in weaker binding between subunits. The specific role for C269 and C272 seems to be to provide −SH groups that are required for octamer formation. A possible role for reduced cysteine residues in maintaining the oligomeric state has been reported for other proteins (Mingorance et al., 1996; Li et al., 1996). It should also be added that we are currently studying a homologous *β*-CA from the unicellular alga *Coccomyxa* (Hiltonen et al., 1995) which we have found to be a tetramer. This enzyme has a tryptophan and a methionine, respectively, at the positions corresponding to C269 and C272 of pea CA (unpublished results).

The altered quaternary structure in the C269−C272 mutants might have induced other, not necessarily extensive, structural changes as well. The spectral properties indicate that the mutations have induced changes in the environments around the tryptophans. The near-UV CD spectra of the wild-type and the C269A mutant are entirely different. In this wavelength region, both the sign and the magnitude of the CD bands depend on the immediate structural and electronic environment of the aromatic chromophores, mainly of the tryptophans. Obviously, there must be surfaces involved in subunit interactions in the octamer that become exposed to the solvent in the tetramer. There are probably also minor conformational changes at one or both tryptophans, while the overall folding of the subunit is still intact. This argument is supported by our finding that the near-UV CD spectrum of the tetrameric *Coccomyxa* CA is very similar both in sign and in intensity to the spectra of the C269−C272 mutants (unpublished results). The intrinsic tryptophan fluorescence intensities also indicate that the environments of the tryptophans have changed as a consequence of the C269−C272 mutations, leading to a situation in which energy transfer between a Trp and a quencher is no longer possible. The fluorescence spectra also show that none of the tryptophans are part of a subunit interface that becomes exposed to the solvent in the tetrameric state. If either of the Trp residues were positioned at such a surface, we would see a red-shifted spectrum in the tetramer mutants, but we do not. Instead, it seems that the hydrophobic surroundings of the tryptophans are largely unchanged. The C269−C272 mutations obviously induce some kind of structural change that decreases the exposure of the remaining cysteines. DTNB is unable to react with any of the remaining cysteines in the C269−C272 mutants, while two and three Cys are accessible in the C165A mutant and the wild-type, respectively. In this context, we note that in the wild-type enzyme, after oxidation by diamide, all −SH groups become inaccessible to reaction with DTNB.

We also have to consider the active site structure. The affinity of sulfonamides to the C269−C272 mutants is weaker than to the unmutated enzyme, but the binding is still strong with dissociation constants below 20 μM. In α-CAs, sulfonamides are known to coordinate directly to the zinc ion, which retains its tetrahedral geometry, with the amide nitrogen replacing the zinc-bound water and acting as a transition-state analogue (Liljas et al., 1994). If we assume that the sulfonamides bind similarly to the *β*-CAs, it means that the zinc-binding area of the active site has to be essentially intact in the mutants, and thus a drastic conformational change of the subunits seems unlikely. The very low catalytic activities of the C269−C272 mutants could result from minor perturbations of the active site, not involving the zinc area. Strong sulfonamide binding, despite a drastically reduced k_{cat} value, has also been reported for some HCA II mutants. In these mutants, parts of the essential hydrogen-bond network of the active site have been disrupted (Liang et al., 1993), or the hydrophobic side of the active site, where the CO_2 substrate molecule presumably binds, has been altered (Fierke et al., 1991). In neither case was it possible to conclude which part of the catalyzed reaction had been affected. Similarly, we cannot determine from our data which step (or steps) in the catalytic mechanism has been most perturbed in the C269−C272 mutants.

What can we say about the individual properties of C269 and C272? Both seem to be necessary for the quaternary structure. From the redox-dependence of the catalytic activity, we find that the C272A mutant is 5−10 times more active, but also more dependent on reducing agents, than the C269A mutant. Thus, the sulfhydryl group at position 269 is more sensitive to oxidation. We note that C269 is conserved among all higher plant CAs, while C272 is absent from the dimeric CA of monocotyledons.

In conclusion, the cysteines at positions 269 and 272 are not involved in direct redox control of the catalytic activity. Instead, the sulfhydryl groups seem to be important for

maintaining the fully functional, native subunit structure. This structure is necessary both for assembly of the subunits to octamers and for the catalytic activity.

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