Subunit interaction in extracellular superoxide dismutase: Effects of mutations in the N-terminal domain

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Abstract

Human extracellular superoxide dismutase (hEC-SOD) is a secreted tetrameric protein involved in protection against oxygen free radicals. Because EC-SOD is too large a protein for structural determination by multidimensional NMR, and attempts to crystallize the protein for X-ray structural determination have failed, the three-dimensional structure of hEC-SOD is unknown. This means that alternative strategies for structural studies are needed. The N-terminal domain of EC-SOD has already been studied using the fusion protein FusNN, comprised of the 49 N-terminal amino acids from hEC-SOD fused to human carbonic anhydrase (HCAII). The N-terminal domain in this fusion protein forms a well-defined three-dimensional structure, which probably contains α -helical elements and is responsible for the tetramerization of the protein.

In this work, we have extended the studies, using site-directed mutagenesis in combination with size-exclusion chromatography, CD, and fluorescence spectroscopy, to investigate the nature of the tetrameric interaction. Our results show that the hydrophobic side of a predicted amphipathic α -helix (formed by residues 14–32) in the N-terminal domain is essential for the subunit interaction.

Keywords: carbonic anhydrase II; extracellular superoxide dismutase; heterologous expression; site-directed mutagenesis; subunit interaction; tetramer contact area

Mammalian cells produce two different types of CuZnSODs, an intracellular and an extracellular form, which are almost equally efficient as catalysts and have nearly identical kinetic parameters (Tibell et al., 1987). The two enzyme forms exhibit significant sequence similarity, but they differ in several respects. The intracellular human CuZnSOD contains 153 amino acid residues per subunit, has a subunit molecular mass of 16 kDa, and is a dimer. The mature human EC-SOD subunit, on the other hand, contains 222 amino acid residues (plus an 18-residue-long signal peptide) and a N-linked oligosaccharide, resulting in a calculated molecular mass of 26.7 kDa (24.2 kDa from the polypeptide and 2.5 kDa from the oligosaccharide) (Edlund et al., 1992). The hEC-SOD protein forms a tetramer, is secreted, and binds to proteoglycans, which are found on the surface of almost every cell in the human body. The central part of the hEC-SOD sequence is homologous with the sequence of the intracellular CuZnSODs, including those amino acid residues that are metal ligands. The 48 N-terminal

amino acid residues of hEC-SOD, and 30 of the C-terminal, have no counterparts in intracellular CuZnSODs, and no significant homology with any other sequenced protein.

The three-dimensional structure of the intracellular CuZnSOD is well known (Tainer et al., 1982), but that of hEC-SOD has eluded determination. However, because the amino acid sequence of the central part of hEC-SOD is highly homologous to CuZnSOD, the structure of this part of hEC-SOD is expected to be very similar to the structure of CuZnSOD.

In a recent report (Tibell et al., 1996), we investigated the functional role of the N-terminal domain of EC-SOD by studying this domain (separate from the other parts of EC-SOD), fused to a monomeric and totally unrelated protein; human carbonic anhydrase II. We showed that the N-terminal domain from hEC-SOD forms a well-defined three-dimensional structure, which probably contains α -helical elements, and that the domain induces formation of a tetrameric fusion protein. However, the nature of the subunit interaction was still unknown.

Subunit interfaces in dimeric and tetrameric proteins have been analyzed and shown to follow general principles (Miller, 1989). These interfaces may be viewed as a combination of four simple structural motifs: (1) extended β -sheets (sheets that form across the interface), as in Concanavalin A (Reeke et al., 1975) and prealbumin (Blake et al., 1978); (2) sheet-sheet packing, as in

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Abbreviations: CuZnSOD, Cu- and Zn-containing intracellular superoxide dismutase; EC-SOD, extracellular superoxide dismutase; GuHCl, guanidine hydrochloride; HCAII, human carbonic anhydrase II; MRW, mean residue weight; SOD, superoxide dismutase.

glyceraldehyde-3-phosphate dehydrogenase (Biesecker et al., 1977); (3) loop interactions, as in triose phosphate isomerase (Banner et al., 1975); and (4) helix-helix packing, as in manganese superoxide dismutase (Borgstahl et al., 1992) and phosphofructokinase (Evans & Hudson, 1979). A structural motif able to carry out an interaction of the latter type might be α -helical coiled coil-like structures that form α -helical bundles.

Our aim for the present investigation was to determine the nature of the tetramer contact of hEC-SOD. We chose a strategy in which we mapped the subunit interaction interface by studying effects of 12 different mutations in the N-terminal domain of our model fusion protein FusNN. The subunit interaction appears to be unusually strong in hEC-SOD because the tetrameric state is not disrupted by 5 M GuHCl or 8 M urea (unpubl. results). We therefore decided to introduce aspartate on the hydrophobic side of the α -helix to maximize the destabilization of the putative helix-helix interaction.

Results

Predictions of secondary structure

According to five different methods for predicting secondary structure (Rost et al., 1994), about 40% of the 49 N-terminal amino acid residues of hEC-SOD might be involved in an α -helical structure (Table 1). As can also be seen in Table 1, they all predict α -helix for the region from amino acid residues 14 to 32. A helical wheel representation (Fig. 1) clearly shows that the α -helix is amphipathic, having one hydrophobic and one hydrophilic side. Our hypothesis is that the hydrophobic side of this α -helix is involved in the subunit interaction. The α -helix (Figs. 1, 2) includes two tryptophans and one tyrosine residue (Trp 16, Trp 28, and Tyr 21), which are located at the transitions between the hydrophobic and hydrophilic sides of the α -helix.

Introduction of mutations in the predicted α -helix

To test our hypothesis experimentally, we used the fusion protein FusNN, and constructed 12 point mutants (Table 1). Five of the

Fig. 1. Helical wheel representation of amino acids (residues 14–31) predicted to adopt α -helical conformation in the N-terminal domain of EC-SOD. Amino acids D12, S13, M32, Q33, and R34 are not predicted to participate in the helix, but, if they were, their relative position would be as presented.

amino acid residue substitutions were made on the hydrophobic side of the predicted α -helix, at positions 17, 20, 24, 27, and 31 (I17D, M20D, V24D, I27D, and V31D, respectively) (Figs. 1, 2). Three of the substitutions, D12A, S13D, and R34A, were introduced at positions outside the predicted α -helix (Table 2), for the following reasons. Because residue 12 is an aspartate in the wild-type protein, it might stabilize the α -helix through N-capping and charge. Introducing an aspartate residue in position 13 might introduce helix stabilization of the same kind as above, whereas Arg 34 may participate in the helix macrodipole stabilization. Three of the mutants, W16A, W28A, and W16A/W28A, were made to find out if any of the tryptophan residues in the N-terminal domain

Table 1. Predictions of α -helical secondary structures in the N-terminal domain of EC-SOD, FusNN, and mutants according to Rost et al. (1994)^a

Protein	Secondary structure prediction of amino acids 1–49
hEC-SOD	WTGEDSAEPNSDS AEWIRDMYAKVTEIWQEV MQRRDDGTLHAACQVQP
FusNN	MAGEDSAEPNSDS AEWIRDMYAKVTEIWQEV MQRRDDGTLHAACQVQP
D12A	MAGEDSAEPNSD SA AEWIRDMYAKVTEIWQEV MQRRDDGTLHAACQVQP
S13D	MAGEDSAEPNSD DA AEWIRDMYAKVTEIWQEV MQRRDDGTLHAACQVQP
W16A	MAGEDSAEPNSDS AE A IRDMYAKVTEIWQEV MQRRDDGTLHAACQVQP
I17D	MAGEDSAEPNSDS AEWDR DMYAKVTEIWQEVMQRRDDGTLHAACQVQP
M20D	MAGEDSAEPNSDS AEWIRDD YAKVTEIWQEVMQRRDDGTLHAACQVQP
A22D	MAGEDSAEPNSDS AEWIRDMYDK VTEIWQEVMQRRDDGTLHAACQVQP
V24D	MAGEDSAEPNSDS AEWIRDMYAKD TEIWQEVMQRRDDGTLHAACQVQP
I27D	MAGEDSAEPNSDS AEWIRDMYAKVTEIWQEV MQRRDDGTLHAACQVQP
W28A	MAGEDSAEPNSDS AEWIRDMYAKVTEIA QEVMQRRDDGTLHAACQVQP
V31D	MAGEDSAEPNSDS AEWIRDMYAKVTEIWQED MQRRDDGTLHAACQVQP
R34A	MAGEDSAEPNSDS AEWIRDMYAKVTEIWQEV MQ AR DDGTLHAACQVQP
W16A/W28A	MAGEDSAEPNSDS AE A IRDMYAKVTEIA QEVMQRRDDGTLHAACQVQP

^a α -Helical structures are shown bold text. Mutations are underlined.

Fig. 2. Model structures of the “wild type” and the five amino acid residue substitutions on the hydrophobic side of the predicted α -helix. Amino acids are colored with a spectrum ranging from blue (charged/polar residues) through white to red (nonpolar residues). Substitutions with aspartate are colored in yellow. The upper row shows a side view of the helix from N-(top) to C-terminal. The lower row shows a top view of the corresponding helices looking from the N-terminal end.

is involved in the tetramer interface. Further, these mutants were used as controls to help us in our interpretation of the fluorescence and CD spectra. Finally, amino acid residue 22 was targeted, because it is positioned in the middle, and at the charged-polar side, of the predicted α -helix (Fig. 1). Aspartate was introduced at this position (A22D) to investigate the effect of an extra negative charge

at the polar face of the α -helix. The assumption was that if the hydrophobic side is engaged in the subunit interaction, an extra charge on the opposite side would have little or no effect. According to the predictions of secondary structure (Table 1), none of the 12 substitutions would disrupt the α -helix.

The mutant fusion proteins were produced in *Escherichia coli* and purified to homogeneity by affinity chromatography as described earlier (Tibell et al., 1996), and the identity of the mutant proteins was verified by DNA-sequencing analysis.

Table 2. Distributions of oligomerization states for FusNN and mutants based on size-exclusion chromatography on Superose 12^a

Fusion protein	Monomer (%)	Dimer (%)	Tetramer (%)
FusNN (wt)	0	0	100
D12A	0	0	100
W16A	3	0	97
A22D	0	0	100
S13D	15	0	85
I17D	30	0	70
R34A	12	0	88
M20D	80	20	0
V24D	60	40	0
I27D	35	65	0
W28A	69	31	0
V31D	40	60	0
W16A/W28A	64	36	0

^aPeaks containing fusion proteins were identified by carbonic anhydrase activity.

Size-exclusion chromatography

The effect of the amino acid residue substitutions on the tetramerization were analyzed by analytical size-exclusion chromatography on Superose 12, and the results are summarized in Table 2. The mutants can be divided into three groups on the basis of results obtained from these studies. Three mutants, D12A, W16A, and A22D, showed little or no difference in oligomerization compared to FusNN. Three other the mutants, S13D, I17D, and R34A, showed a distribution between monomers and tetramers. The mutants M20D, V24D, I27D, W28A, W16A/W28A, and V31D showed a distribution between monomers and dimers. None of the mutations led to the formation of a pure monomeric form.

CD spectra

CD spectra of the mutated proteins in the far- and near-UV region were recorded and compared to the corresponding spectra for FusNN and HCAII. As can be seen in Figure 3A, B, and C, the near-UV CD spectra of the mutated proteins and FusNN exhibit the same CD fine structures as the HCAII spectrum. The slightly higher

Fig. 3. Near-UV spectra of FusNN, mutants, and HCAII with accompanying curve lines. **A:** FusNN (—); HCAII (-----); D12A (---); S13D (- - - -); A22D (- - -); R34A (- - -). **B:** FusNN (—); HCAII (-----); I17D (---); M20D (—); V24D (-----); I27D (- - -); V31D (- - -). **C:** FusNN (—); HCAII (-----); W16A (- - -); W28A (-----); W16A/W28A (- - -).

intensity of FusNN, and the markedly lower intensity of the mutants S13D and R34A cannot be explained currently. However, the tryptophan residues in the N-terminal domain apparently do not add any intense band to the spectra.

The CD spectra in the far-UV region of HCAII, FusNN, and the mutants are shown in Figure 4A, B, and C. The contributions from

Fig. 4. Far-UV spectra of FusNN, mutants, and HCAII with accompanying curve lines. **A:** FusNN (—); HCAII (-----); D12A (---); S13D (- - - -); A22D (- - -); R34A (- - -). **B:** FusNN (—); HCAII (-----); I17D (---); M20D (—); V24D (-----); I27D (- - -); V31D (- - -). **C:** FusNN (—); HCAII (-----); W16A (- - -); W28A (-----); W16A/W28A (- - -).

the N-terminal domain to the various far-UV CD spectra were obtained by calculating the difference between the mutant spectra and the HCAII spectrum. The resulting difference spectra are shown in Figure 5A, B, and C. Secondary structure predictions based on these difference spectra, according to the variable selection method developed by Manavalan and Johnson (1987), are shown in Table 3.

Fig. 5. Difference spectra in far-UV. These spectra were obtained after subtracting the spectrum in molar ellipticities of HCAII from the spectra of the fusion proteins. Resulting spectra were recalculated to mean residue weight ellipticity for the N-terminal domain. **A:** FusNN (—); D12A (---); S13D (-----); A22D (- - -); R34A (- - -). **B:** FusNN (—); I17D (- - -); M20D (—); V24D (-----); I27D (- - -); V31D (- - -). **C:** FusNN (—); W16A (- - -); W28A (-----); W16A/W28A (- - -).

The A22D mutant displayed difference spectrum with intense negative bands around 210 nm, a less intense negative band around 222 nm, and intense positive bands at 192 nm (Fig. 5A). This spectrum is similar to that of FusNN, which indicates that this mutant

Table 3. Secondary structure predictions from the difference CD-spectra in far-UV using the variable selection method of Manavalan and Johnson (1987)

N-terminal	α -Helix	Antiparallel β -sheet	Parallel β -sheet	Turn	Other
FusNN	36	11	4	20	30
A22D	38	10	4	22	27
D12A	31	20	6	15	28
S13D	25	22	4	20	30
W16A	20	18	7	25	31
R34A	23	19	1	23	33
I17D	5	25	6	27	38
M20D	3	25	3	29	40
V24D	5	27	2	22	43
I27D	4	24	4	27	41
W28A	5	28	5	24	38
V31D	3	21	0	25	50
W16A/W28A	4	29	4	21	40

does not differ significantly from FusNN in structure. Secondary structure prediction analysis of these spectra (Table 3) suggests a large α -helix content for FusNN, 36%, and A22D, 38%. The difference spectra of the mutants D12A, S13D, and W16A also show positive peaks, with maxima at 192 nm (Fig. 5A,C) and negative bands around 210 and 222 nm that are not as intense as for FusNN. This implies a slight decrease in α -helical content, to 31, 25, and 20% respectively, according to the predictions (Table 3). Despite a quite different shape of the far-UV CD spectrum of R34A (Fig. 5A), the secondary structure prediction indicates a 23% α -helix content in this mutant.

The difference CD spectra in far-UV of the mutants I17D, M20D, V24D, I27D, W28A, W16A/W28A, and V31D are more difficult to interpret (Fig. 5B). These CD spectra do not display any positive bands in the 190–200-nm region, and all of them have negative bands around 210 and 222 nm, which are not as intense as those of FusNN. The secondary structure predictions based on these spectra clearly show that the mutations have, directly or indirectly, induced changes in the secondary structure (e.g., α -helical content) of the N-terminal domain. The α -helix content of these mutants is only 3–5%, compared to 36% for FusNN (Table 3).

Fluorescence

The fluorescence emission maximum (λ_{max}) of a protein resulting from the excitation of tryptophan residues is related to their environment, with residues in apolar environments having blue-shifted, or shorter wavelength emissions than those in hydrophilic environments. The fluorescence intensity depends on many factors, such as energy transfer to other tryptophan residues or to nearby internal or external quenchers.

Fluorescence emission spectra of the mutants were recorded from 300 nm to 450 nm, after excitation at 295 nm, and compared to the spectrum of FusNN and HCAII. The spectrum of FusNN has an emission maximum around 334 nm, which is about the same (or slightly red-shifted) as for HCAII (Fig. 6, inset). However, the intensity of the fluorescence is stronger for FusNN, indicating that

Fig. 6. Difference fluorescence spectra (fusion protein-HCAII) showing the fluorescence emission deriving from the N-terminal domain. (—), FusNN, and mutants displaying monomer-tetramers; (-----), mutants displaying monomer-dimers, and tryptophan mutants (— · —). The inset graph shows the original spectra of FusNN and HCAII.

one or both of the tryptophan residues in the fused N-terminal domain is not quenched.

To examine the fluorescence spectra of the N-terminal domain, the spectrum of HCAII was subtracted from the FusNN spectrum and from the mutant spectra. The resulting difference spectra, shown in Figure 6, give information about environmental changes around Trp 16 and Trp 28 in the N-terminal domain resulting from the different mutations.

Discussion

The N-terminal domain of hEC-SOD, when fused to HCAII, is responsible for the tetramerization of the fusion protein, FusNN, (Tibell et al., 1996). It is most probable, therefore, that it also performs this function in hEC-SOD. The secondary structure predictions based on the primary structure of the 49 amino acid residues of the N-terminal domain (Table 1) correlate well with the predictions based on the difference far-UV CD spectrum between HCAII and FusNN (Table 3), and they indicate an α -helix content of 40 and 36%, respectively. The involvement of the α -helix in the subunit interaction is therefore most probable. A structural motif able to carry out such a tetramer interaction, involving α -helices, might be a four-helix bundle. Examination of amino acid sequences present in α -helical coiled coils and α -helical bundles reveals the common presence of segments containing heptad repeats (*abcdefg*) with hydrophobic residues at positions *a* and *d*. There is no simple rule for assessing the significance of short heptad runs in a sequence. However, according to current structural knowledge, Cohen and Parry (1990) claim that runs of two to three heptads, which have at least 80% hydrophobic occupancy of the *a* and *d* positions, have high probability for helix formation. Such α -helices are amphipathic in nature and are driven together in hydrophilic environments, forming hydrophobic interfaces in α -helical coiled coils or α -helical bundles. This arrangement, in turn, stabilizes the α -helix. Studies on energetics of packing α -helices in limited bundles have shown that dipole interactions favor antiparallel packing (Hol et al., 1981; Hol, 1985; Shoemaker et al., 1987), but some parallel helix pairs have

been observed and are, for example, essential for dimerization of leucine zippers (O'Shea et al., 1991).

Applying the heptad repeat approach on the predicted α -helix in the N-terminal domain of hEC-SOD reveals two consecutive heptad repeats with 100% hydrophobic occupancy, starting with Ile 17 at an *a* position in the first heptad and ending the second heptad at position *g* with Glu 30. Amino acid residue 31 (valine), in position *a* in a third incomplete heptad repeat, may also participate in the hydrophobic stripe.

Substitutions on the hydrophobic side (positions 20, 24, 27, and 31)

The α -helix is clearly involved in the subunit interactions, according to our results, because the amino acid substitutions at positions 20, 24, 27, and 31, located on the hydrophobic side of the predicted α -helix (Figs. 1, 2), all disrupt the tetrameric interaction (Table 2), as well as leading to a dramatic loss of α -helical elements (Table 3). Further, the fluorescence emission difference spectra of all these mutants display a pronounced red shift and decrease in fluorescence intensities compared to those of the other mutants and FusNN (Fig. 6). This indicates a more polar environment around the tryptophan residues in these mutants.

No contribution to the near-UV CD spectra from the tryptophan residues of the N-terminal domain could be observed in these mutants, indicating that the tryptophan residues are mobile i.e., that they are not in an anisotropic environment (Fig. 3B). Because these mutants have a low α -helix content and they do not form tetramers, it seems likely that the tryptophan residues are, at least partly, shielded from the solvent in the intact tetramer and probably form part of the interface.

Substitutions on the hydrophilic side (positions 12 and 22)

Substitutions in position 12 and 22 (D12A and A22D), on the hydrophilic side of the N-terminal domain (Fig. 1), had no apparent effect on the tetramerization (Table 2), and little or no effect on the secondary structure (Table 3; Fig. 5A). Evidently, the hydrophilic side of the amphipathic α -helix is not involved in the tetrameric interaction. The fluorescence emission difference spectra supports this interpretation, because mutant A22D is approximately identical to FusNN and the mutant variant D12A (Fig. 6) displays a decrease in intensity, but no significant red-shift. The latter result also indicates that one or both of the tryptophan residues might be quenched by the substitution, but it does not lead to any dramatic structural changes in the environment around Trp 16 and Trp 28.

Possible helix-destabilizing mutants (positions 12, 13, and 34)

The substitutions in positions 12, 13, and 34 are all situated outside the predicted α -helix. These mutants were made partly to investigate stabilizing effects of the α -helix. The proposed N-capping and macrodipole stabilizing function of residue 12 has minor or no importance because the substitution, D12A, decreased the α -helix content only slightly (Table 3) and the variant is 100% tetramer (Table 2). The introduction of an additional macrodipole-stabilizing residue and retaining the N-capping by the mutation S13D rather destabilized the α -helix (Table 3). However, this could be a con-

sequence of substituting the serine residue with aspartate on the hydrophobic side in the very N-terminal part of the amphiphilic α -helix. Arg 34 might have a macrodipole-stabilizing effect, because the mutant R34A exhibited an α -helical content of 23% (Table 3). In summary, substitutions of residue Ser 13 and Arg 34 both resulted in a decrease in α -helix content paralleled by a partial disturbance of the tetrameric interaction (resulting in 85% and 88% tetramer, respectively; Table 2), from which it can be postulated that these amino acid residues lie on the N- and C-terminal borders of the tetramer interface of the α -helix. This conclusion is strengthened by the fact that no significant red-shift was observed in the fluorescence emission difference spectra of these variants (Fig. 6), implying that the mutation induced no dramatic structural changes in the environment of Trp 16 and Trp 28.

Substitution of position 17

The most N-terminal position in the heptad repeat of the predicted α -helix is Ile 17. The variant with a substitution at this position, I17D, is noteworthy because it, like the other mutants with substitutions on the hydrophobic side of the α -helix, has a very low α -helix content (5%; Table 3). However, in contrast to those variants, but like other N-terminal substitutions (i.e., those at position 12 and 13), it results in only partial disturbance of tetramer formation, giving 70% tetramer (Table 2). The latter observation suggests that the tetramer interface is located more toward the C-terminal end of the α -helix.

Substitution of the tryptophan residues in the N-terminal domain (positions 16 and 28)

FusNN contains nine tryptophan residues. Seven are in the HCAII part of the fusion protein. The other two, Trp 16 and Trp 28, are located in the predicted α -helix of the fused N-terminal domain, positioned at the transitions between the hydrophobic and hydrophilic side of the α -helix (Figs. 1, 2). Because of the hydrophobic nature of these aromatic amino acid residues, they might help in shielding the proposed hydrophobic tetramer contact area. If this is the case, these tryptophan residues might be used to give information about the degree of structural changes in their vicinity. The substitutions W16A, W28A, and W16A/W28A were made to clarify the role of the N-terminal tryptophan residues.

The substitution in position 16 had essentially no effect on either the tetramerization (Table 2), the secondary structure of the N-terminal (Table 3), or on the fluorescence spectrum (Fig. 6). In contrast, the substitution of Trp 28 disrupted the α -helical structure (Table 3), as well as abolishing the tetramerization (Table 2). The effect was observed in both mutants involving a substitution at position 28 (W28A and W16A/W28A). These results indicate that Trp 28 is essential, whereas Trp 16 has no crucial function for the tetramer interaction.

When Trp 16 was replaced by alanine, the difference-fluorescence spectrum was hardly changed at all, whereas the substitution in position 28 (W28A) caused a large decrease (about 50%) of the corresponding spectrum. Interestingly, all other mutants with no tetramer contact have red-shifted difference spectra, supporting that the environment of Trp 28 is part of the tetramer interface. These results indicate that Trp 16 must be quenched in the tetrameric proteins (FusNN and W16A) and that the contribution of Trp 28 to the spectrum is dominating.

Summary

The HCAII part of the fusion protein is essentially unaffected by fusion of the N-terminal domain. It has already been shown that the catalytic activity of FusNN equals that of HCAII (Tibell et al., 1996). We now have additional evidence for this conclusion because the variant where both of the tryptophan residues were displaced (W16A/W28A) showed a fluorescence emission spectrum (Fig. 6) and a CD spectrum in the near-UV region (Fig. 3C) almost identical to the corresponding HCAII spectra.

The results from our 12 mutations all support that residues 17–31 participate in the formation of an α -helix of an amphiphatic nature, that the hydrophobic side of this α -helix (positions 20, 24, 27, 28, and 31) is involved in the subunit interaction, and that the α -helical structure is stabilized by the tetrameric interaction. The latter conclusion is strengthened by the fact that all amino acid substitutions that disrupt the tetrameric interaction also lead to a dramatic loss of α -helical elements, although none of these substitutions are predicted to disturb the α -helical structure (Table 1). Further, results from the tryptophan mutants, as well as from many of the other mutants, suggest the C-terminal part of the predicted α -helix (residues 20–31) to be most important for the tetramer formation.

There are four possible ways whereby the hydrophobic side of the predicted α -helix could avoid a hydrophilic environment: (1) by interacting with hydrophobic parts of the fusion protein (HCAII); (2) by interacting with other hydrophobic patches in the N-terminal domain; (3) by interacting with the HCAII part of another subunit of the fusion protein; and (4) by interacting with the N-terminal domain of another subunit. Because no hydrophobic patch is present in either HCAII or in other parts of the N-terminal domain, the most probable interpretation is alternative 4. The four helices, one from each subunit, could be arranged either parallel or antiparallel. The results in our study cannot be used to favor either of the possibilities.

Materials and methods

Construction and purification of the fusion proteins

The construction of an expression plasmid (p ACNN), coding for FusNN, is described in Tibell et al. (1996). To construct the 12 mutants, we used site-directed mutagenesis essentially according to the method of Kunkel (1985), with the slight modification described in Mårtensson et al. (1995). Oligonucleotides were synthesized on Applied Biosystems 392 DNA/RNA Synthesizer. All plasmids were prepared using WizardTM Miniprep DNA purification system from Promega. Mutations were confirmed by DNA sequencing of the entire coding region, using the chain-termination method of Sanger et al. (1977). Fusion proteins were produced and purified as described earlier for various carbonic anhydrase variants (Khalifah et al., 1977; Mårtensson et al., 1995).

Analysis of fusion proteins

The CO₂-hydration activity of HCAII was measured according to Rickli et al. (1964). N-terminal amino acid sequences were determined using an Applied Biosystems model 477A sequencing system. Protein extinction coefficients were determined according to the method of Gill and von Hippel (1989), which is based on the amino acid residue composition of each protein and the measured

absorption values at 280 nm for the native protein, and for the protein denatured in 6 M GuHCl.

SDS-PAGE was performed on a discontinuous polyacrylamide gel system (Jergil & Ohlsson, 1974) containing 14.9% acrylamide and 0.1% SDS. For western blotting, the proteins were transferred to an Immobilon-P membrane by standard methods. Blots were developed using highly purified rabbit anti-EC-SOD or rabbit anti-HCAII and alkaline phosphatase-labeled anti-rabbit IgG antibodies.

Size-exclusion chromatography

The apparent molecular mass of each of the native fusion proteins was estimated by chromatography on a Superose 12 HR 10/30 column (Pharmacia Biotech), using 10 mM potassium phosphate, pH 7.6, and 0.15 M NaCl as eluant, an elution rate of 0.5 mL/min, an injection volume of 200 μ L, and protein concentrations of 2 mg/mL. The column was calibrated with following standards: thyroglobulin (670 kDa), bovine immunoglobulin G (158 kDa), ovalbumin (44 kDa), equine myoglobin (17.5 kDa), and vitamin B12 (1.35 kDa). For mutant V24D, three different protein concentrations were used (1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL). No concentration dependence of the oligomeric distribution could be seen, indicating that the different oligomeric species is determined upon folding in the cell.

CD

CD spectra were recorded at 23 °C on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France) essentially as described by Freskgård et al. (1994). Far-UV spectra for the proteins were recorded in a 0.5-mm quartz cell at a protein concentration of 0.5–0.7 mg/mL in 5 mM sodium phosphate, pH 7.6. Near-UV CD spectra were recorded in a 5.0-mm quartz cell using the same buffer and protein concentration. Difference spectra, representing the CD signal originating from the N-terminal domains, were obtained by subtracting the HCAII spectra in molar ellipticities from the spectra of the fusion proteins. The resulting difference spectra were recalculated to give mean residue weight ellipticities for the N-terminal domains (i.e., 53 amino acid residues, comprising 49 from the hEC-SOD N-terminal domains and 4 from the spacer). Secondary structure predictions of the difference spectra were performed by the variable selection method, described in detail by Manavalan and Johnson (1987).

Fluorescence measurements

Fluorescence emission spectra were obtained using a Shimadzu RF-5000 spectrofluorophotometer at 25 °C in a 10 $\times$ 4 mm cuvette. The protein concentration was 0.5 μ M and the pH was kept at 7.5 using a 25 mM sodium phosphate buffer. All samples were excited at 295 nm when measuring intrinsic fluorescence; tyrosine and phenylalanine residues have negligible absorbances at this wavelength, and the emitted light therefore will originate from the tryptophan residues. When measuring the intrinsic fluorescence, the bandwidths for excitation and emission were set to 5 and 3 nm, respectively, and the emission spectra were recorded between 300 and 450 nm. All spectra were corrected by subtracting the spectrum of the blank, which lacked the enzyme but was otherwise identical to the sample. The fluorescence spectrum of HCAII was

subtracted from each of the fluorescence emission spectra of the fusion proteins. The resulting difference spectra therefore represent the fluorescence characteristics of the various N-terminal domains.

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