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The cupredoxin fold is found in the soluble Cu_A and CyoA domains of two terminal oxidases

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Abstract

The CD spectra of the Cu_A domain from subunit II of *Paracoccus* cytochrome *c* oxidase and the CyoA domain of subunit II from *E. coli* quinol oxidase have been recorded in the wavelength region 260–185 nm. A computer program based on a set of CD spectra of proteins with known structures, and employing the stastistical method of variable selection, has been used to estimate the distribution of five forms of secondary structure. The analysis was improved by including the CD spectra of azurin and plastocyanin in the basis set. For the Cu_A domain, an estimate from the primary structure was also made. The results show that the soluble domains have the cupredoxin fold, with very little helical structure and a predominance of β-strands. The CyoA domain is very similar to azurin, but the β-structure in the Cu_A protein resembles that in plastocyanin.

Key words: Cytochrome oxidase; Quinol oxidase; Azurin; Plastocyanin; Circular dichroism; Protein secondary structure

1. Introduction

Cytochrome *c* oxidase and quinol oxidase are two terminal respiratory enzymes, which belong to a heme-copper oxidase superfamily [1,2]. The primary acceptor of electrons from cytochrome *c* is a binuclear Cu_A site [3,4]. This is located outside the membrane in subunit II in bacterial as well as in mitochondrial cytochrome *c* oxidases. The soluble domain containing this site in *Paracoccus* oxidase has recently been expressed and shown by EPR to be closely related to the native redox center [5]. Quinol oxidase from *E. coli* has a homologous subunit II, which, however, lacks the ligands for Cu_A [1,2,6]. The C-terminal CyoA domain of this subunit has been expressed as a soluble protein, and it has, in addition, been possible to restore the lost metal-binding site by designing a mutant protein with the metal ligand amino acids in the correct positions in the sequence [6]. These findings suggest that the soluble domains retain the secondary structure of the native proteins. Since subunit II is homologous also with blue copper proteins, such as azurin and plastocyanin [1,2], it is likely that this structure is the cupredoxin fold, a Greek key β-barrel found as a structural motif in small blue copper proteins as well as in a subdomain in multicopper oxidases [1,2,7]. In fact, structural models of the Cu_A site have been constructed [1,8] on the basis of the known structures of azurin [9] and plastocyanin [10].

As a basis for further modelling of the Cu_A domain, we have studied the secondary structure of it and the CyoA domain by circular dichroism (CD). The structure prediction from the CD spectra was made with a computer program [11–13]. The accuracy of the estimate was considerably improved by including the CD spectra of azurin and plastocyanin, with their known X-ray structures, among the proteins in the basis set. For the Cu_A domain, a secondary structure prediction was also made from the amino acid sequence [5]. Our results show that the two soluble domains have secondary structures closely resembling those of small blue proteins, particularly azurin, with very little α-helix and a predominance of β-structure. The sequence data place the short helical segments close to the C-terminus, just as in azurin [9].

2. Materials and methods

2.1. Proteins

The soluble domains of subunit II of the two oxidases were prepared as described [5,6]. Wild-type *Pseudomonas* azurin and spinach plastocyanin were purified as in [14,15]. For calculating the concentrations of protein, the following millimolar absorption coefficients at 278 nm were used: 48.7 $mM^{-1} \cdot cm^{-1}$(Cu_A domain), 23.6 $mM^{-1} \cdot cm^{-1}$ (CyoA domain), 8.8 $mM^{-1} \cdot cm^{-1}$ (azurin) and 5.4 $mM^{-1} \cdot cm^{-1}$ (plastocyanin). The absorbancies were measured in 1-cm cells in a Hitachi U-1100 spectrophotometer; with azurin and plastocyanin the measurements were made with approximately 20 times more concentrated solutions than used for recording the CD spectra.

2.2. CD and optical spectra

CD spectra were recorded in a Jasco J720 spectropolarimeter at 20°C in the wavelength region 260–185 nm. The conditions employed were based on recommendations in [16]. A 1-mm cell was used, and the cell compartment was continuously purged with N_2 to avoid absorption from O_2. All measurements were made in 0.5 mM potassium phosphate buffer, pH 7.4. The protein concentration was about 0.05 mg/ml, and the total optical absorbance of buffer, protein and cell never exceeded 0.6 even at the lowest wavelengths. A slit width of 2 nm was used; other instrumental settings are listed in Table 1. All spectra were the average

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of 10 recordings. CD data are expressed as differential molar absorption coefficients ($\Delta\varepsilon = \varepsilon_l - \varepsilon_r$) per amino acid residue, based on the following number of amino acids: 155 (Cu_A domain), 213 (CyoA domain), 128 (azurin) and 99 (plastocyanin).

Optical spectra of the subunit II domains were recorded in a Cary 4 UV-visible spectrophotometer in the wavelength range 350–900 nm with a 1-cm cell, and were identical to those published [5,6]. The absorption of the samples used for CD spectra was measured against air in the same 1-mm cell in the range 260–185 nm.

2.3. Computational methods

Most computer programs for estimating secondary structures from CD data are based on calculated reference spectra for represenative structures, but the program used here [11–13] takes a different approach. Instead of calculating reference spectra, the method analyzes the CD spectrum of a protein from the CD spectra of 22 proteins with known structure in a basis set [11]. The program was improved [12] by the introduction of the statistical method of variable selection [17]. In addition, we could further increase the accuracy of the estimate for the subunit II domains by including the CD spectra of azurin and plastocyanin in the basis set.

For the estimation of the secondary structure from the amino acid sequence, a network procedure developed by Rost and Sander was used [18]. The prediction is performed by a system of neural networks, and the input is a multiple sequence alignment. In the case of the Cu_A domain, a comparison was made with the sequences of subunit II from 69 different species.

3. Results and discussion

The CD spectra of the Cu_A and CyoA domains are shown in Fig. 1. To improve the secondary structure prediction by the computer program used [12], we also recorded the CD spectra of azurin and plastocyanin, whose secondary structures are known accurately from high-resolution X-ray structures [9,10], to include in the basis set of proteins; these spectra are given in Fig. 2. Table 2 summarizes the results of the estimation of five forms of secondary structures in the two subunit II domains and compares them with the established structures in azurin and plastocyanin. A prediction from the amino acid sequence [18] of the Cu_A domain is also included in the table. It can be seen that within the errors estimated, the two estimates agree with each other (even if the sequence method only distinguishes between three types of secondary structure). The CD spectrum of the CyoA domain had been measured earlier but not analyzed in the same detail as here [6]. It was concluded that there

Table 1
Instrumental settings for A = Azurin and Plastocyanin, Cu_A domain, and CyoA domain

Proteins	Wavelength region (nm)	Data point intervals (nm)	Scan speed ($nm \cdot min^{-1}$)	Time constants (s)
A	260–200	0.2	20	1
Cu_A domain	260–200	0.2	10	2
CyoA domain	260–200	0.5	20	1
A	200–185	0.2	5	4
Cu_A domain	200–185	0.2	2	8
CyoA domain	200–185	0.5	5	4

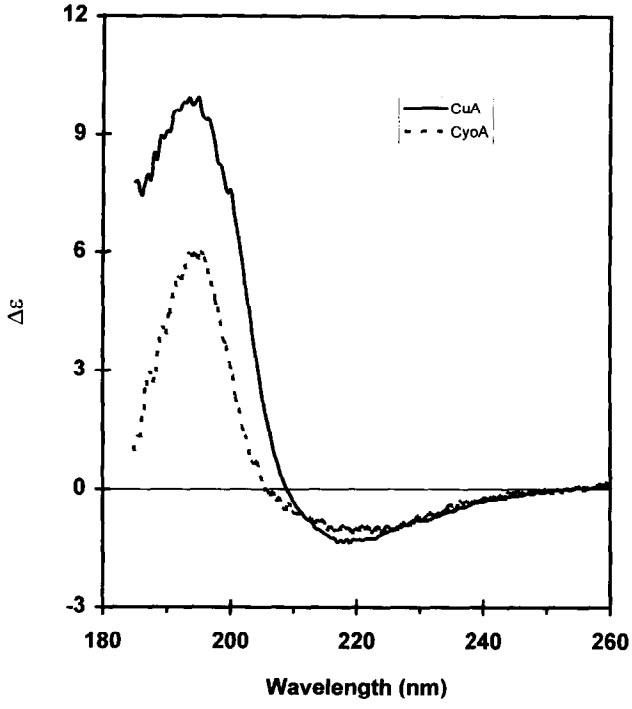

Fig. 1. CD spectra of the Cu_A and CyoA domain.

is very little α-helix, which agrees with the present result. The amount of β-sheet, on the other hand, was stated to be as high as 70%, whereas our estimate is 44%. Including also the β-turns, we find 66% β-structure, however, in close agreement with the earlier prediction.

Table 2 shows that the percentage distribution of the five forms of secondary structure in the subunit II do-

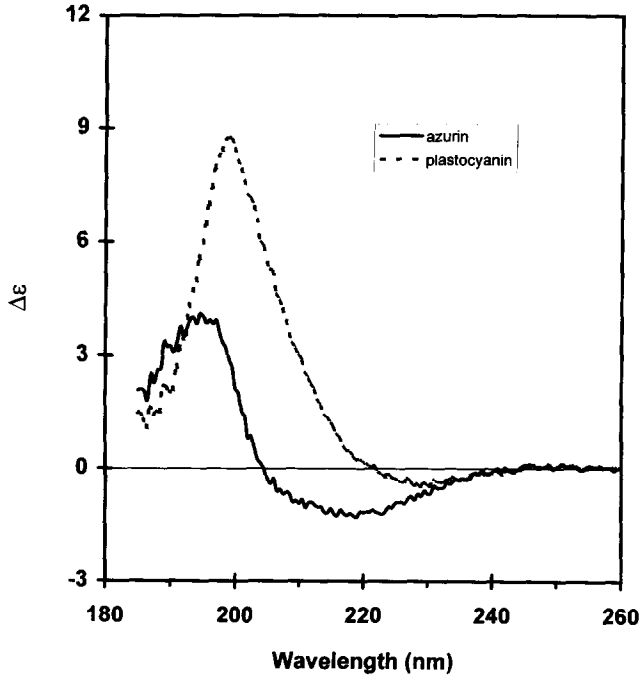

Fig. 2. CD spectra of azurin and plastocyanin.

Table 2
Secondary structure of the Cu_A and CyoA domains, and of azurin and plastocyanin

Protein	Method	α-helix	Anti-parallel β-sheet	Parallel β-sheet	β-turn	Other	Sum
Azurin	X-ray	10	33	11	22	24	100
Plastocyanin	X-ray	4	38	18	28	12	100
Cu_A domain	CD	14 ± 6	31 ± 10	17 ± 6	27 ± 5	12 ± 8	101
	Sequence	10	45		45		100
CyoA domain	CD	10 ± 3	33 ± 4	11 ± 3	22 ± 2	25 ± 4	100

mains is almost identical with that in azurin, despite the longer peptide chains in the two domain structures. This means that the additional amino acids participate in different secondary structures in approximately the same proportions as in the shorter proteins. The CyoA domain is most like azurin, whereas the Cu_A protein bears similarities to plastocyanin in its β-structure. The helical content is, however, closer to that in azurin, both according to the CD and sequence analysis. The sequence prediction suggests that the small amount of helix is found in two stretches of 16 amino acids, separated by 10 residues in strand and loop structures, close to the C-terminal end of the molecule. One of the short helices in azurin is also found near the C-terminus [9].

Two antiparallel β-strands is a common supersecondary structural motif, called a Greek key β-barrel [19]. This is found in small blue proteins, such as azurin and plastocyanin, where it is called the cupredoxin fold [20]. It is also found in the multicopper oxidase, ascorbate oxidase [7]. In all cases, the copper ion is located at the 'northern' end of the molecule. Our results suggest that the cupredoxin fold is present also in the soluble Cu_A domain of cytochrome *c* oxidase as well as in the CyoA domain of quinol oxidase. This may seem surprising in view of the fact that it now appears established [3,4] that Cu_A, unlike type 1 Cu in blue proteins, is a binuclear site. In order to design mutagenesis and electron-transfer experiments with the Cu_A domain, it becomes desirable to construct a new molecular model, including the entire peptide chain and a binuclear Cu site. Our estimation of the secondary structure of the two soluble subunit II domains should assist such efforts considerably.

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