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## Analysis of Protein Circular Dichroism Spectra Based on the Tertiary Structure Classification

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We have developed a method that utilizes the determination of tertiary structure class and creates a tertiary class-specific reference protein set. We have performed protein circular dichroism (CD) analysis using a tertiary class-specific reference set and CDPro software package, which contains three popular methods for CD analysis. Additional flexibility introduced in the analysis by the use of a tertiary class-specific reference protein set results in improvements in the secondary structure estimations of  $\alpha\alpha$  and  $\beta\beta$  classes, with no deterioration in the results for the  $\alpha\beta$  class. The main improvements obtained were for the prediction of  $\alpha$ -helical and  $\beta$ -strand fractions of  $\alpha\alpha$  and  $\beta\beta$  proteins.

CD is a widely used technique for studying peptide and protein conformations. One of the most successful applications of CD, the structural characterization of proteins, depends upon the remarkable sensitivity of far-UV CD to the backbone conformation of proteins. The basic principle involved in the analysis of protein CD spectra, and used in the estimation of secondary structure fractions, is that the protein CD spectrum ( $C_\lambda$ ) can be expressed as a linear combination of component secondary structure spectra ( $B_{k\lambda}$ ):  $C_\lambda = \sum f_k B_{k\lambda}$ , where  $f_k$  is the fraction of the secondary structure  $k$ . Various methods have been developed to estimate the secondary structure fractions (1–10), and to assign the tertiary structure class (11) of a protein, from the

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analysis of its CD spectrum. Inadequacies of the assumptions involved in the simple relation between the CD spectrum and secondary structure fractions are overcome by the flexibility of the analysis, introduced by variable selection (6, 7, 10) or variable weighting (5) of reference proteins. We have introduced additional flexibility in the analysis by using a tertiary class-specific reference protein set. Tertiary structure class was assigned by the method of Venyaminov and Vassilenko (11), and the secondary structure fractions were estimated using a smaller but tertiary class-specific reference protein set. The computer program CLUSTER, which determines the tertiary structure class and creates the appropriate reference protein set, is included in the CDPro software package (12).

### CLUSTER Program

This program is based on the method of Venyaminov and Vassilenko (11) and determines the tertiary structure class of a given protein from its CD spectrum. The CD spectra of 46 native proteins and seven denatured samples in the range 190–236 nm, at 2-nm intervals, were used to construct a 24-dimensional hyperspace corresponding to the ellipticity values at the 24 wavelengths; each CD spectrum thus forms one point. Proteins belonging to different tertiary structure classes form separate groups, or clusters, in this hyperspace. The equations of the hyperplanes separating the different groups are used to assign any new CD spectrum to one of the five tertiary structure classes ( $\alpha\alpha$ ,  $\beta\beta$ ,  $\alpha + \beta$ ,  $\alpha/\beta$ , and denatured; 13). In our implementation, we combined the  $\alpha + \beta$  and  $\alpha/\beta$  classes to form an  $\alpha\beta$  class, because of the similarity of the CD spectra of proteins belonging to these two classes.

The CLUSTER program uses the generic input file of the CDPro software package. Upon execution, CLUSTER determines the tertiary structure class of the protein, creates the appropriate reference protein set for subsequent use in the CD analysis programs, and modifies the option in the input file for using the CD and structural data of the tertiary class-specific reference set.

### Reference Proteins

Different sets of reference proteins, with varying numbers of proteins and having a good representation of  $\alpha$ -rich,  $\beta$ -rich, and mixed  $\alpha\beta$  proteins, have been used in the secondary structure analyses. We have constructed a reference set of 47 proteins by combining the unique protein CD spectra from four sources (12). The tertiary class-specific reference protein sets form subsets of this 47-protein reference protein set. Among the 47 proteins, 8 belong to the  $\alpha\alpha$  tertiary class, 11 to the  $\beta\beta$  class, 22 to the  $\alpha\beta$  class, and the remaining 6 to the denatured class. The  $\alpha\beta$  class contains proteins belonging

to  $\alpha + \beta$  and  $\alpha/\beta$  classes, since it is difficult to distinguish the two classes by CD. The proteins and the corresponding crystal structures (PDB code) are listed below. The resolutions of the crystal structures used were better than 2.5 Å, except for structure 1eri (2.7 Å).

*Eight  $\alpha\alpha$  proteins.* Myoglobin (4mbn), hemoglobin (2mhb), hemerythrin (2hmz), T4 lysozyme (2lzm), cytochrome *c* (5cyt), colicin A (1col), insulin (4ins), and parvalbumin (5cpv).

*Eleven  $\beta\beta$  proteins.*  $\alpha$ -Chymotrypsin (5cha), elastase (3est),  $\gamma$ -crystallin (4gcr), prealbumin (2pab), concanavalin A (2ctv), Bence-Jones protein (1rei), tumor necrosis factor (1tnf), superoxide dismutase (2sod),  $\alpha$ -chymotrypsinogen (2cga), green fluorescent protein (1ema), and rat intestinal fatty acid binding protein (1ifc).

*Twenty-two  $\alpha\beta$  proteins.* Triosephosphate isomerase (8tim), lactate dehydrogenase (6ldh), lysozyme (1lys), thermolysin (8tln), phosphoglycerate kinase (3pgk), *EcoRI* endonuclease (1eri), flavodoxin (1fx1), subtilisin BPN' (1sbt), glyceraldehyde-3-phosphate dehydrogenase (1crw), papain (9pap), ribonuclease A (3rn3), pepsinogen (2psg),  $\beta$ -lactoglobulin (1beb), azurin (5azu), alcohol dehydrogenase (8adh), carbonic anhydrase (1ca2), glutathione reductase (3grs), rhodanese (1rhd), carboxypeptidase A (5cpa), bovine pancreatic trypsin inhibitor (5pti), adenylate kinase (3adk), and staphylococcal nuclease (2sns).

Secondary structure assignments from DSSP (14) were used to determine the secondary structure fractions of the globular proteins in the reference set. The  $\alpha$ -helix and  $\beta$ -strand structures were split into regular and distorted classes, considering four residues per  $\alpha$ -helix and two residues per  $\beta$ -strand distorted (9). Our grouping of DSSP assignments gave us six secondary structural classes: regular  $\alpha$ -helix,  $\alpha_R$ ; distorted  $\alpha$ -helix,  $\alpha_D$ ; regular  $\beta$ -strand,  $\beta_R$ ; distorted  $\beta$ -strand,  $\beta_D$ ; turns, T; and unordered, U.

### CD Analysis

Analysis of the CD spectra was carried out using the three methods for analyzing protein CD spectra for secondary structure estimation included in the CDPro software package (12): SELCON3 (8, 9), CONTIN/LL (5), and CDSSTR (10). Each of these methods implements a different algorithm, the details of which have been published. The CD spectrum of the protein analyzed was removed from the reference set and the secondary structure fractions were determined using the other members of the reference set. The performance (RMS deviation and correlation coefficient between the CD-predicted and the DSSP-assigned values) of the three programs with the *all-protein* reference set is compared to that of the *tertiary class-specific* reference set in Table 1.

TABLE 1  
Performance of SELCON3, CDSSTR, and CONTIN/LL Programs, for Analyzing Protein CD Spectra,  
for All-Protein and Tertiary Class-Specific Reference Sets<sup>a</sup>

Protein set <sup>b</sup>	Method <sup>c</sup>	$\alpha_R$		$\alpha_D$		$\beta_R$		$\beta_D$		T		U			
		$\delta$	$r$	$\delta$	$r$	$\delta$	$r$	$\delta$	$r$	$\delta$	$r$	$\delta$	$r$	$\delta$	$r$
$\alpha\alpha$ (8)	SELCON3 (All)	0.034	0.976	0.042	0.204	0.048	0.059	0.036	0.288	0.061	0.635	0.069	0.639	0.050	0.950
	SELCON3 (TC)	0.029	0.978	0.045	-0.458	0.019	-0.306	0.021	0.024	0.048	0.674	0.105	0.015	0.053	0.943
	CONTIN/LL (All)	0.059	0.957	0.057	0.316	0.085	-0.168	0.040	-0.017	0.081	0.122	0.066	0.686	0.066	0.910
	CONTIN/LL (TC)	0.057	0.910	0.040	-0.292	0.024	-0.500	0.024	-0.234	0.074	0.386	0.088	0.364	0.057	0.934
$\beta\beta$ (11)	CDSSTR (All)	0.034	0.965	0.042	0.096	0.061	-0.229	0.035	0.166	0.056	0.585	0.091	0.581	0.057	0.933
	SELCON3 (All)	0.037	0.396	0.053	-0.407	0.097	0.276	0.038	0.164	0.082	-0.478	0.127	-0.144	0.080	0.757
	SELCON3 (TC)	0.027	0.185	0.036	-0.196	0.080	0.565	0.051	-0.342	0.085	-0.289	0.087	0.310	0.065	0.849
	CONTIN/LL (All)	0.052	0.109	0.051	-0.325	0.109	0.035	0.028	0.557	0.088	-0.584	0.071	-0.037	0.072	0.793
$\alpha\beta$ (22)	CONTIN/LL (TC)	0.033	0.160	0.045	-0.623	0.053	0.671	0.039	0.068	0.090	-0.688	0.098	-0.031	0.065	0.855
	CDSSTR (All)	0.043	0.135	0.043	-0.005	0.094	-0.113	0.025	0.542	0.063	-0.021	0.049	0.534	0.057	0.874
	SELCON3 (All)	0.065	0.708	0.042	0.434	0.057	0.630	0.023	0.569	0.068	0.054	0.058	0.315	0.054	0.793
	SELCON3 (TC)	0.057	0.734	0.055	0.131	0.056	0.520	0.034	-0.006	0.065	0.123	0.061	0.279	0.056	0.778
41	CONTIN/LL (All)	0.064	0.743	0.043	0.429	0.053	0.708	0.023	0.600	0.073	-0.058	0.064	0.227	0.056	0.787
	CONTIN/LL (TC)	0.067	0.654	0.041	0.294	0.059	0.547	0.025	0.484	0.080	-0.174	0.065	0.032	0.059	0.744
	CDSSTR (All)	0.071	0.727	0.044	0.418	0.060	0.626	0.026	0.517	0.068	0.088	0.075	-0.003	0.060	0.748
	CDSSTR (TC)	0.082	0.656	0.041	0.428	0.060	0.530	0.026	0.529	0.082	-0.061	0.079	-0.126	0.065	0.706
	SELCON3 (All)	0.056	0.936	0.045	0.751	0.070	0.788	0.027	0.825	0.072	0.356	0.060	0.562	0.057	0.860
	SELCON3 (TC)	0.046	0.958	0.049	0.778	0.059	0.863	0.037	0.674	0.068	0.367	0.079	0.348	0.058	0.863
	CONTIN/LL (All)	0.059	0.931	0.046	0.733	0.070	0.795	0.025	0.849	0.074	0.286	0.065	0.495	0.059	0.853
	CONTIN/LL (TC)	0.058	0.931	0.042	0.828	0.052	0.890	0.029	0.804	0.082	0.234	0.080	0.269	0.060	0.851
CDSSTR (All)	0.062	0.928	0.042	0.813	0.070	0.789	0.027	0.835	0.064	0.482	0.072	0.496	0.058	0.856	

<sup>a</sup>  $\delta$ , root mean square deviation;  $r$ , correlation coefficient;  $\alpha_R$ , regular  $\alpha$  helix;  $\alpha_D$ , distorted  $\alpha$  helix;  $\beta_R$ , regular  $\beta$  strand;  $\beta_D$ , distorted  $\beta$  strand; T, turns; U, unordered.

<sup>b</sup>  $\alpha\alpha$  (8), 8 proteins in the  $\alpha\alpha$  class;  $\beta\beta$  (11), 11 proteins in the  $\beta\beta$  class;  $\alpha\beta$  (22), 22 proteins in the  $\alpha\beta$  class; 41, 41 proteins from  $\alpha\alpha$ ,  $\beta\beta$ , and  $\alpha\beta$  classes.

<sup>c</sup> The CD analysis program used with the all-protein (All) or tertiary class-specific (TC) reference set. The number of proteins in the all-protein reference set was 47. Only the  $\alpha\beta$  class is large enough to provide an adequate class-specific reference set for CDSSTR.

CD analysis with SELCON3 and CONTINLL using the tertiary class-specific reference set gives slightly better performance indices than that from the all-protein reference set. Full analysis could not be performed with CDSSTR because the small number of proteins in the  $\alpha\alpha$  and  $\beta\beta$  tertiary class-specific reference protein sets did not give a sufficient number of random combinations required for a valid solution.

Improvements were obtained with the tertiary class-specific reference set in the prediction of  $\alpha$ -helical and  $\beta$ -strand fractions of  $\alpha\alpha$  and  $\beta\beta$  proteins. This is probably because of the limited and specific information content provided by the  $\alpha\alpha$  and  $\beta\beta$  tertiary class-specific reference sets. It is known that proteins belonging to the  $\alpha\alpha$  tertiary class have little or no  $\beta$ -sheet structure (15). Introduction of spectral and structural information for the  $\beta$ -sheet in the analysis of an  $\alpha\alpha$  tertiary class-specific protein may have a negative effect on the analysis. A similar situation exists for proteins belonging to the  $\beta\beta$  tertiary class that have little or no  $\alpha$ -helical structure.

Results obtained for  $\alpha\beta$  proteins with the tertiary class-specific reference set were similar to those obtained with the all-protein reference set.  $\alpha\beta$  proteins have both  $\alpha$ -helical and  $\beta$ -sheet structures and the

spectral variations are well represented in both all-protein and  $\alpha\beta$  tertiary class-specific reference sets. The variable selection procedure implemented in the CD analysis programs counters the negative effects of extreme values of  $\alpha$ -helix or  $\beta$ -sheet content, present in  $\alpha\alpha$  and  $\beta\beta$  proteins in the all-protein reference set, on the analysis of  $\alpha\beta$  proteins.

In summary, we have developed a method that utilizes the determination of tertiary structure class and creates a tertiary class-specific reference protein set for protein CD analysis. The flexibility introduced by the variable selection or variable weighting of the reference proteins has resulted in significant improvements in protein CD analyses. A reference protein set composed of proteins belonging to the tertiary structure class of the analyzed protein provides additional flexibility and a better basis for the analysis. We performed CD analysis using the tertiary class-specific reference set and CDPro software package, which contains three popular methods for CD analysis. We obtained improvements in the secondary structure estimations of  $\alpha\alpha$  and  $\beta\beta$  classes with no deterioration in the results for the  $\alpha\beta$  class. The computer program CLUSTER, which determines the tertiary structure class and cre-

ates the appropriate reference protein set for the analyzed protein, is included in the CDPro software package, which is available online at <http://lamar.colostate.edu/~sreeram/CDPro>.

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## Detection of Antibacterial Polypeptide Activity *in Situ* after Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

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Antimicrobial polypeptides play an important role in the immune defense mechanisms in the animal kingdom. To date several types of polypeptide antibiotics such as cecropins, defensins, attacins, etc., from animal sources were identified (1, 2). The methods used for estimation of antimicrobial polypeptides activity are based mainly on growth inhibition zone assay on agar plates (3) or on the liquid growth inhibition microassays (4). Native polyacrylamide gel electrophoresis in acidic (pH 4.3) conditions (5) with subsequent bioassay (6, 7) is useful technique for the studies on cationic polypeptides as well.

In this paper we describe very sensitive and reproducible procedure for antibacterial polypeptides activity restoration after electrophoretic separation on polyacrylamide gels in denaturing conditions. The method was adapted for both glycine–SDS/PAGE according to Laemmli (8) and for tricine–SDS/PAGE developed by Schägger and von Jagow (9), routinely used for separation of proteins according to their molecular mass. Our studies were performed in parallel on the synthetic cecropin B (Sigma), derived from sequence analysis of cecropin B purified from *Hyalophora cecropia*, and on *Galleria mellonella* (*Lepidoptera*) immune hemolymph. For *G. mellonella* immunization, *Escherichia coli* lipopolysaccharide (Sigma) in the amount of 2.5  $\mu\text{g}$ /seven-stage larvae was used and hemolymph was collected after 48 h. The presence of antibacterial activity, induced by immunization, was detected by growth inhibition zone assay on agar plates. Samples containing 0.1–2.0  $\mu\text{g}$  of cecropin B or 25–100  $\mu\text{g}$  of total hemolymph protein, as determined by Bradford method (10), were separated by electrophoresis in polyacrylamide gels as described in the original procedures (8, 9). The gels were then washed in 2.5% Triton X-100 (Bio-Rad) for 30 min for removal of SDS. Polypeptides in the gels were renatured by washing in 50 mM Tris–HCl, pH 7.5, and subsequently in the Luria broth (Difco), each step for 30 min. To localize the polypep-

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