Analysis of the Circular Dichroism Spectra Pattern of the Disulfide Bonds in RNase T_1 and Its Mutants*

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Abstrak

Analisis mengenai struktur suatu protein, dapat dilakukan dengan berbagai cara, antara lain dengan menganalisis spektrum dikroisme sirkular (circular dichroism spectra, CD spectra)nya. Dengan menganalisis CD spectra suatu protein, dapat diperkirakan kandungan struktur α heliks, β sheet, dan random coil pada protein tersebut. Para peneliti telah menggunakan CD spectra untuk menganalisis ikatan disulfida pada beberapa protein, namun belum dapat mengambil kesimpulan tentang ciri pola CD spectra suatu ikatan disulfida. Pada penelitian ini kami meneliti pola CD spectra ikatan disulfida pada RNase T_1 dan mutannya, yang mempunyai satu, dua dan tiga ikatan disulfida, dengan maksud memperkaya perbendaharaan pola CD spectra. Untuk itu kami meneliti CD spectra dua macam protein yang mempunyai dua ikatan disulfida, yaitu protein alam RNase T_1 (mempunyai ikatan disulfida antara C2-C10, C6-C103) dan protein mutan, yaitu RNase $T_1S_{(C2S, C10S)}$ (mempunyai ikatan disulfida antara C24-C84, C6-C103); satu macam protein mutan yang mempunyai tiga ikatan disulfida, yaitu RNase T_1S (mempunyai ikatan disulfida antara C2- C10, C24-C84, C6-C103); dan satu macam protein mutan yang hanya mempunyai satu ikatan disulfida, yaitu RNase T_1 (C2S, C10S) (hanya mempunyai ikatan disulfida antara C6-C103). Keempat macam protein tersebut disintesis dengan menggunakan cara rekayasa genetika dan protein. CD spectra diukur menggunakan alat CD (JASCO J-500A, Nihon Wako), pada suhu 25°C, dengan konsentrasi protein 0,1 mg/ml, dalam akuades, untuk analisis spektrum antara 200-235nm, dan konsentrasi protein 0,2 mg/ml, dalam 10mM buffer kalium fosfat pH 7,5, untuk analisis spektrum antara 235-310nm. Dari gambaran CD spectra keempat macam protein tersebut, kami mengambil kesimpulan, bahwa pola CD spectra ikatan disulfida tidak menunjukkan ciri yang jelas, dan polanya dapat berbeda tergantung letak ikatan disulfida yang bersangkutan; namun pada RNase T_1 dan mutannya, ikatan disulfida menunjukkan puncak-puncak dikroik positif lemah di sekitar 235-285nm.

Abstract

A protein's structure can be analyzed using several methods, e.g. using the circular dichroism spectra (CD spectra) of the protein. Analyzing the CD spectra of a protein can help us to predict the α helix, β sheet and random coil content of the protein. To analyze the disulfide bonds in certain proteins, some investigators used the CD spectra, but could not come to a conclusion about the specific CD spectra pattern of a disulfide bond. In this research, we analyzed the CD spectra pattern in RNase T_1 and its mutants, which had one, two and three disulfide bonds to enrich the CD spectra data. For this purpose we analyzed the CD spectra of two proteins, each had two disulfide bonds, ie. the wild type RNase T_1 (having disulfide bonds between C2-C10, C6-C103) and the mutant protein, RNase $T_1S_{(C2S,C10S)}$ (having disulfide bonds between C24-C84, C6-C103); a mutant protein having three disulfide bonds, ie. RNase T_1S (having disulfide bonds between C2-C10, C24-C84, C6-C103); and a mutant protein having only one disulfide bond, ie. RNase T_1 (C2S,C10S) (having a disulfide bond between C6-C103). The four proteins were synthesized using the genetic and protein engineering technique. CD spectra were measured using CD equipment (JASCO J-500A, Nihon Wako), at 25 C. Spectrum analysis between 200-235nm, was done using 0.1 mg/ml protein in aquadest, and spectrum analysis between 235-310nm, was done using 0.2 mg/ml protein in 10mM potassium phosphate buffer, pH 7.5. The CD spectra of the four proteins lead us to the conclusion that the CD spectra pattern of a certain disulfide bond does not have a specific pattern, and the pattern can differ according to the location of the disulfide bond in the protein; but in RNase T_1 and its mutants, the disulfide bond shows weak positive dichroic bands between 235-285nm.

Keywords: *Circular dichroism spectra (CD spectra), Disulfide bond, RNase T_1*

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INTRODUCTION

An accurate analysis of the conformation (three dimensional structure) of a protein can only be done by X-ray crystallography. This is a laborious and complicated method, requiring X-ray diffraction studies of suitable protein crystals. There are various methods in making protein crystals, depending on the protein to be crystallized, and most are laborious and time consuming. In addition to the difficulties encountered in making the protein crystals, X-ray crystallography needs a sophisticated and expensive diffractometer and computer system to collect and analyze the data. There are other methods to analyze the conformation of a protein but not as accurate as X-ray crystallography, such as the predictive method based on the amino acid sequence, which requires complicated computations, or based on the results of the optical rotatory dispersion (ORD) or circular dichroism (CD) spectra recordings of the protein.[1]

Several analysis of protein conformations using their circular dichroism (CD) spectra have been reported.[2-4] Greenfield and Fasman (1969) showed that the prediction of the α helix, β sheet, and random coil content of a protein using its CD spectra patterns between 208-240 mμ, is more accurate than using its ORD spectra patterns. This conclusion is made after comparing the results based on CD spectra recordings and the results of X-ray crystallography, which were very much in line.[2] Several analysis of the disulfide bonds in proteins have also been reported, but until recently there are no conclusion about the characteristics of the CD spectra pattern of the disulfide bond, because the search of these characteristics is based on the dichroic bands which were not due to the aromatic side chains such as tyrosine, tryptophan or phenylalanine.[1,3,5] Therefore we analyze the CD spectra pattern of the wild type RNase T_1 (having two disulfide bonds) and three kinds of its mutants (having one, two and three disulfide bonds), in order to get the characteristics of the CD spectra of the disulfide bond, to enrich the data of the CD spectra pattern.

MATERIALS AND METHOD

Materials

The wild type RNase T_1 (having disulfide bonds between C2-C10 and C6-C103) was a gift from Sankyo (Tokyo, Japan), and the three kinds of its mutants, ie. RNase $T_{1(C2S,\ C10S)}$ (having one disulfide bond between C6-C103), RNase $T_1S_{(C2S,\ C10S)}$ (having disulfide bonds between C24-C84 and C6-C103), and RNase T_1S (having disulfide bonds between C2-C10, C24-C84 and C6-C103) (Figure 1) were produced in the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan, using the genetic and protein engineering method.

The enzymes used in genetic and protein engineering were purchased from Takara Shuzo (Kyoto, Japan) or New England Biolabs (Beverly, MA, USA).

All materials were of reagent grade and were obtained from commercial sources as described previously.[6] Oligodeoxyribo-nucleotides were synthesized in an Applied Biosystem synthesizer, and CD spectra were recorded on a Jasco J-500A spectropolarimeter (Nihon Wako).

Production of the three RNase T_1 mutants

The construction of the genes for the three RNase T_1 mutants was performed by ligating the chemically synthesized oligodexyribo-nucleotides.[6] Expression of the mutant genes were performed in recombinants of *E. coli* HB 101 which harbored the mutant genes inserted plasmids, having a Trp promoter and an alkaline phosphatase signal peptide genes, upstream of the mutant genes. By this method, the mutant proteins were sufficiently produced and easily purified, because the proteins were expressed in the periplasmic region of the *E. coli*. Recovery of the proteins were performed by the osmotic shock method using solutions of 25% sucrose, 1 mM EDTA and 30 mM Tris-HCl, pH 8. The proteins were purified by Q-Sepharose and Sephadex G-50 column chromatography.[7,8]

Circular dichroism spectroscopy

The CD spectra of RNase T_1 and its mutants were recorded on a Jasco J-500A spectropolarimeter, at 25°C. The CD spectra measurements in the 200-235 nm region were performed at 0.1 mg protein/ml, in H_2O, using a cell of 1mm width, and the measurements in the 235-310 nm region were performed at 0.2 mg protein/ml, in 10 mM potassium phosphate, pH 7.5, using a cell of 10 mm width.[7,8]

Analysis of the CD spectra patterns

The CD spectra patterns of RNase T_1 and its mutants were transfered on a graph paper, and computations were performed to get the CD spectra patterns of the

C2-C10 and C24-C84 disulfide bonds. The CD spectra patterns of the disulfide bond between C2-C10 were computed by substracting the pattern of the wild type RNase T_1 by that of RNase $T_{1\,(C2S,\,C10S)}$, and substracting the pattern of RNase T_1S by that of RNase $T_1S_{(C2S,\,C10S)}$, and the CD spectra patterns of the disulfide bond between C24-C84 were computed by substracting the pattern of RNase T_1S by that of RNase T_1, and substracting the pattern of RNase $T_1S_{(C2S,\,C10S)}$ by that of RNase $T_{1\,(C2S,\,C10S)}$.

RESULTS AND DISCUSSION

Analysis of protein CD spectra in the 200-260 region have been reported to show the prediction of the α helix, β sheet, and random coil content, hence the conformation of the protein.[2-4]

In the 200-260 nm region, RNase T_1 and its mutants showed almost a same CD spectra pattern (Figure 2a), thus the conformations of the four proteins were almost similar. In the 230-330 nm region, the dichroic bands can be attributed to the aromatic side chains and disulfide bonds.[1] In the 230-330 nm region, RNase T_1 and its mutants showed similar patterns but differed in the magnitude of the dichroic bands (Figure 2b). RNase T_1 and its mutants have the same aromatic side chains and the only difference is in the content of the disulfide bond, therefore the difference in the magnitude of the dichroic bands in the 230-330nm region, reflects the CD spectra of the disulfide bond.

Figure 3 shows the CD spectra patterns of the disulfide bonds between C2-C10, which was derived from the differences in the CD spectra patterns between RNase T_1S and RNase $T_1S_{(C2S,\,C10S)}$ (Figure 3a), and RNase T_1 and RNase $T_{1\,(C2S,\,C10S)}$ (Figure 3b); figure4 shows the CD spectra patterns of the disulfide bonds between C24-C84, which was derived from the differences in the CD spectra patterns between RNase $T_1S_{(C2S,\,C10S)}$ and RNase $T_{1(C2S,\,C10S)}$ (Figure4a), and RNase T_1S and RNase T_1 (Figure 4b); and Figure 5 shows the CD spectra patterns of the disulfide bonds between C2-C10 and their average (Figure 5a), and the CD spectra patterns of the disulfide bonds between C24-C84 and their average (Figure 5b).

Figure 3a, 3b and 5a shows that the CD spectra pattern of the C2-C10 disulfide bond has weak positive dichroic bands at 237 nm, 280 nm and 305 nm. This finding is close to that of Saxena and Wetlaufer[3] which showed that the disulfide bond had dichroic bands in a region between 260 and 300 nm. Moreover, Sander and T'so[5] suggested that in RNase T_1, the dichroic band at 235 nm might be due to a disulfide bond. Figure 4a, 4b and 5b shows that the CD spectra pattern of the C24-C84 disulfide bond has positive dichroic bands at 250 nm and 273 nm, and a negative dichroic band at 302 nm. This finding is in line with that of Sander and T'so[5] who found positive dichroic bands at 235 nm and 250 nm, and a negative dichroic band at 305 nm, in RNase T_1, which might be due to the disulfide bonds. In this research, the dichroic bands due to the disulfide bonds are weak and indistinct, hence the CD spectra

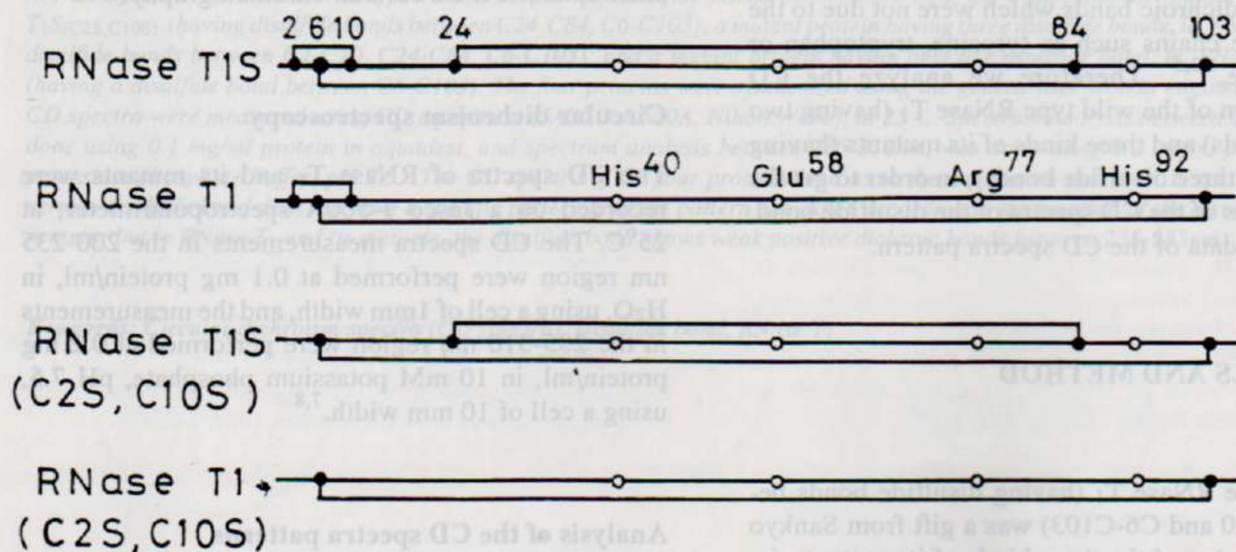

Figure 1. Schematic alignment of the disulfide bonds in RNase T_1 and its mutants.
Closed circles indicate cystein, and open circles indicate active site residues : His40, Glu58, Arg77 and His92.

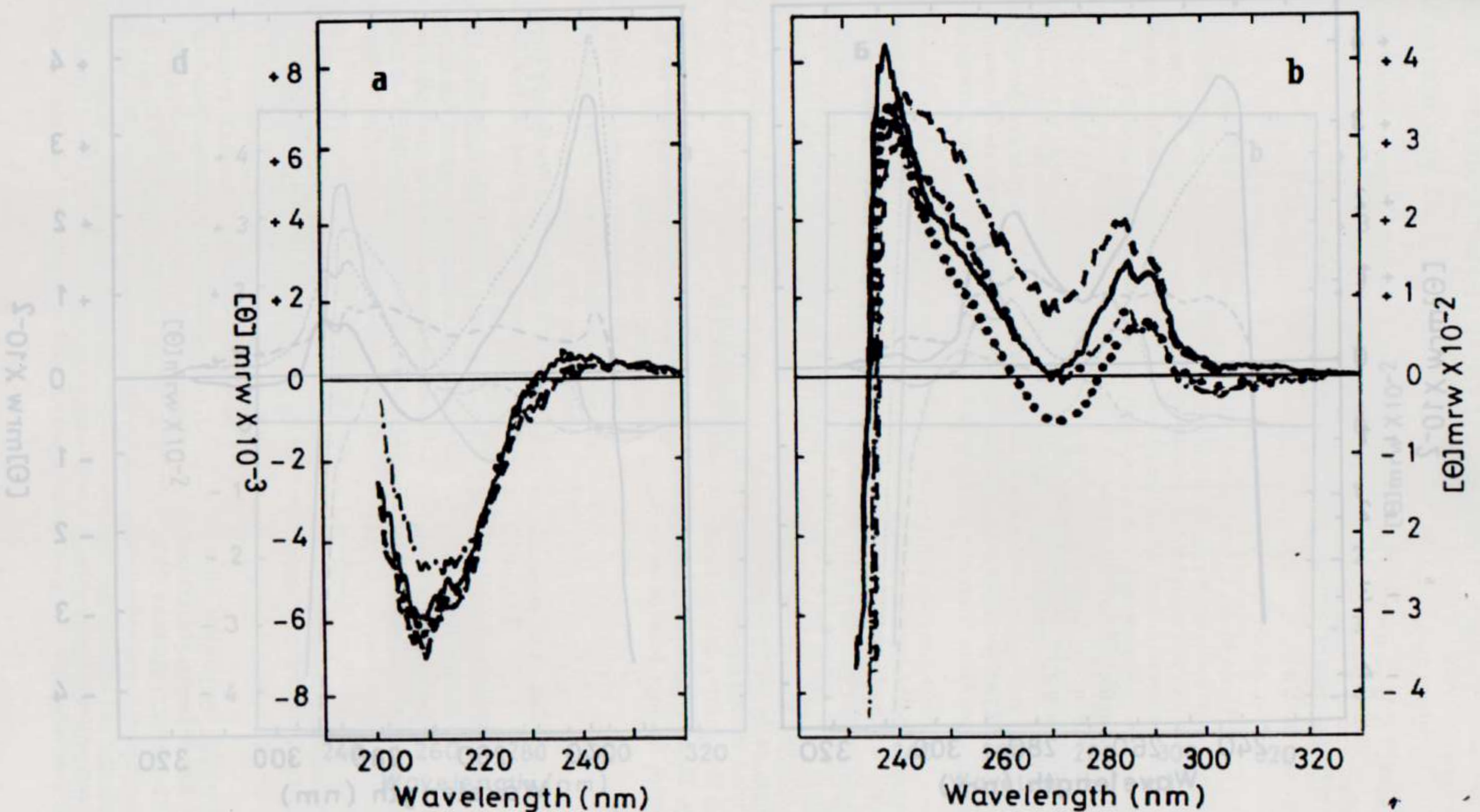

Figure 2. The CD spectra patterns of RNase T_1 and its mutants.
Wild type RNase T_1 (——), RNase T_1S (– – –),
RNase $T_{1(C2S,\ C10S)}$ (· · · · ·), and RNase $T_1S_{(C2S,\ C10S)}$ (– · – · –).
(a) measured at 25°C, in H_2O, at 0.1 mg protein/ml.
(b) measured at 25°C, in 10 mM potassium phosphate, pH 7.5, at 0.2 mg protein/ml.

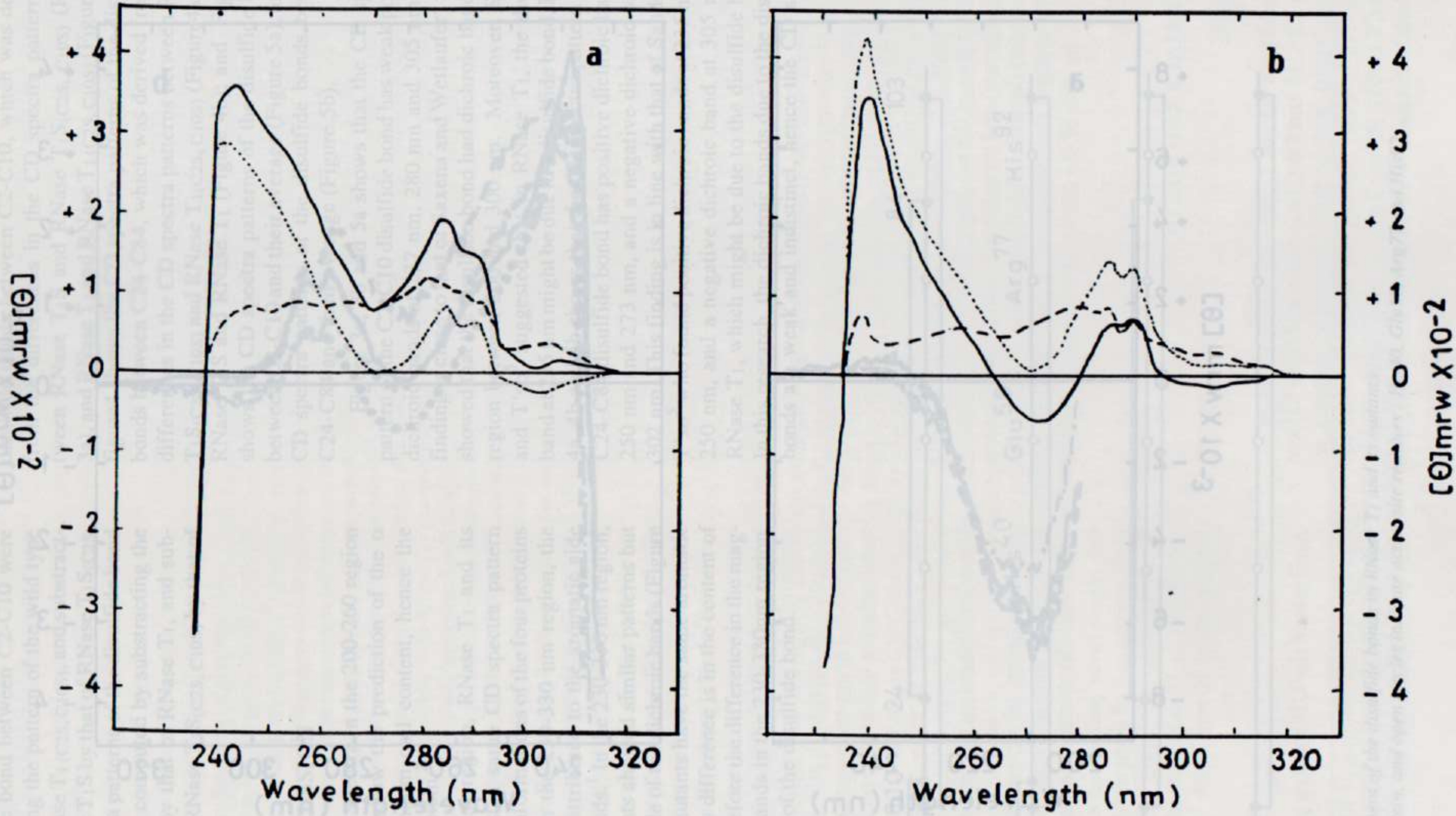

Figure 3. The CD spectra patterns of the C2-C10 disulfide bonds (- - -).
(a) computed by substracting the pattern of RNase T_1S (——) by that of RNase $T_1S_{(C2S, C10S)}$ (· · · · ·).
(b) computed by substracting the pattern of RNase T_1 (· · · · ·) by that of RNase $T_{1\ (C2S, C10S)}$ (——).

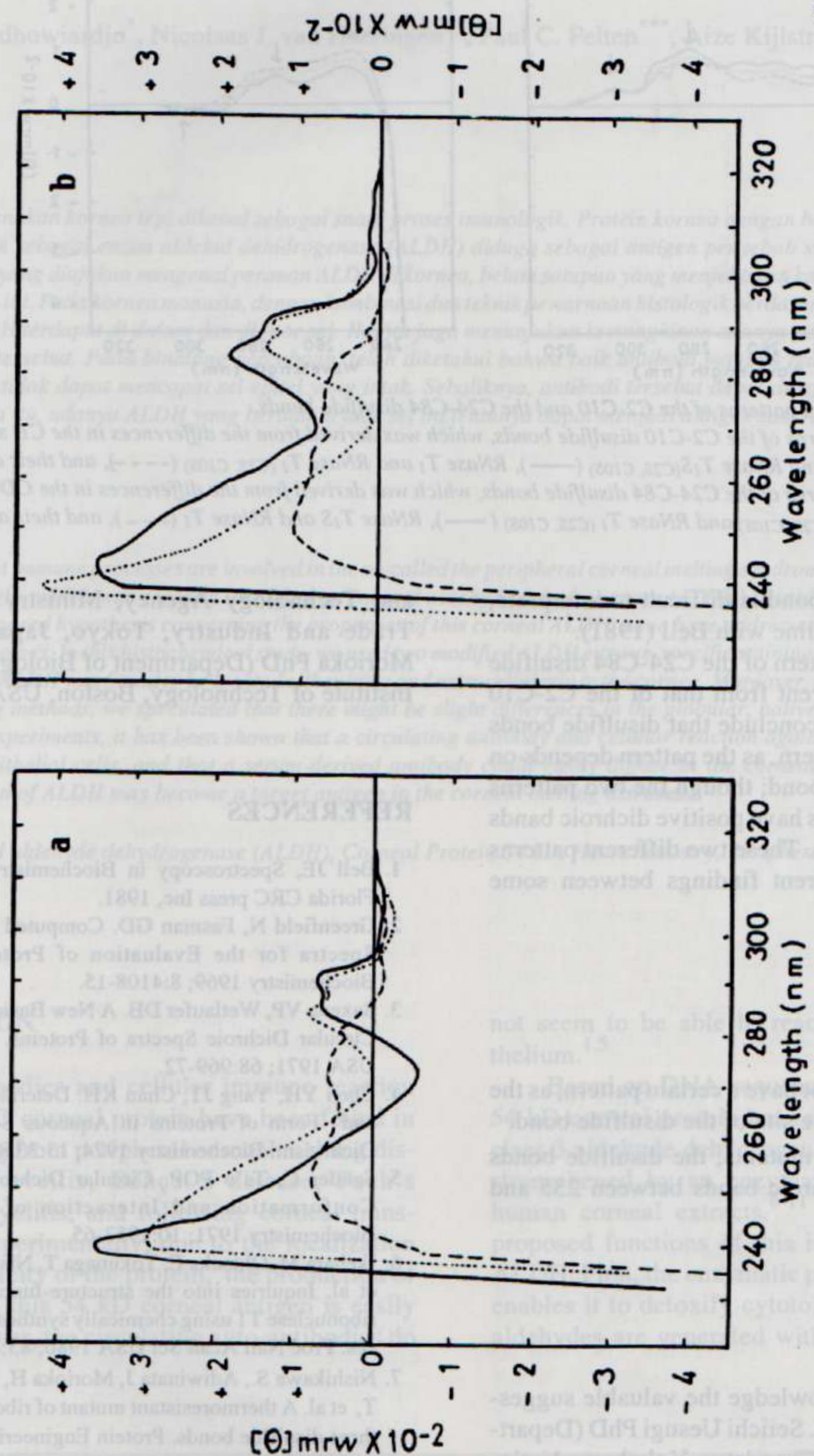

Figure 4. The CD spectra patterns of the C24-C84 disulfide bonds (— —). (a) computed by substracting the pattern of RNase $T_1S_{(C2S, C10S)}$ (· · · ·) by that of RNase $T_{1\,(C2S, C10S)}$ (——). (b) computed by substracting the pattern of RNase T_1S (——) by that of RNase T_1 (· · · ·).

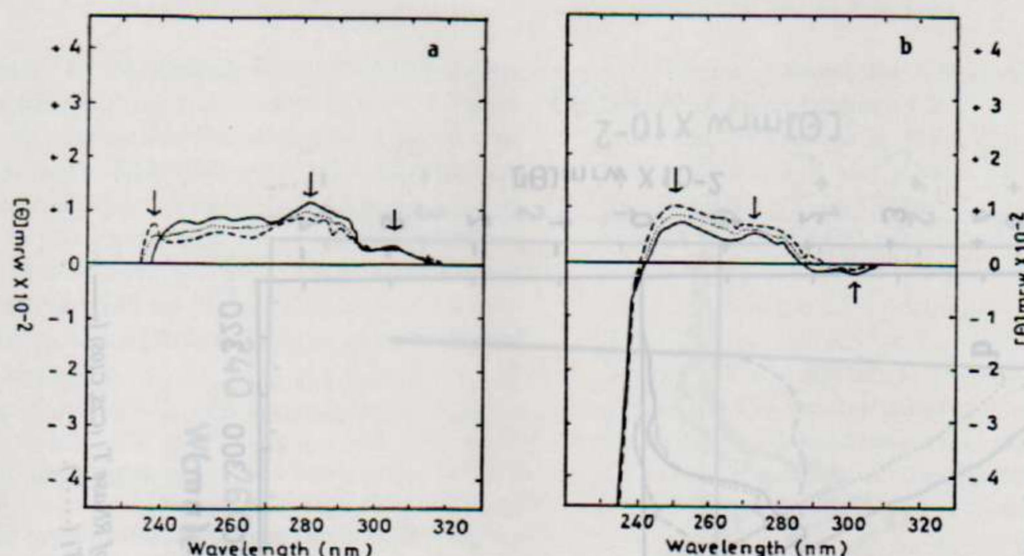

Figure 5. The CD spectra patterns of the C2-C10 and the C24-C84 disulfide bonds.
(a) the CD spectra patterns of the C2-C10 disulfide bonds, which was derived from the differences in the CD spectra patterns between RNase T_1S and RNase $T_1S_{(C2S,\ C10S)}$ (——), RNase T_1 and RNase $T_{1\ (C2S,\ C10S)}$ (- - - -), and their average (· · · · ·).
(b) the CD spectra patterns of the C24-C84 disulfide bonds, which was derived from the differences in the CD spectra patterns between RNase $T_1S_{(C2S,\ C10S)}$ and RNase $T_{1\ (C2S,\ C10S)}$ (——), RNase T_1S and RNase T_1 (- - -), and their average (......).

pattern of the disulfide bond is difficult to interprete, and this is very much in line with Bell (1981).[1]

The CD spectra pattern of the C24-C84 disulfide bond is a little bit different from that of the C2-C10 disulfide bond, thus we conclude that disulfide bonds do not have a certain pattern, as the pattern depends on the site of the disulfide bond; though the two patterns show that disulfide bonds have positive dichroic bands between 235 and 285 nm. These two different patterns might explain the different findings between some researchers.[1,3,5]

CONCLUSION

1. Disulfide bonds do not have a certain pattern, as the pattern depends on the site of the disulfide bond.
2. In RNase T_1 and its mutants, the disulfide bonds showed positive dichroic bands between 235 and 285 nm.

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