# CIRCULAR DICHROISM STUDIES ON CHEMICALLY MODIFIED DERIVATIVES OF CONCANAVALIN A

William D. McCUBBIN, Kimio OIKAWA and Cyril M. KAY
*Department of Biochemistry, University of Alberta, Edmonton, Canada*

Received 22 February 1972

## 1. Introduction

Earlier observations [1,2] revealed that native Con A at pH 5.2 and 7.0 possessed a definite fine structure in the CD spectral region of aromatic absorption, i.e., 250–320 nm. The involvement of both tyrosine and tryptophan chromophores in the generation of these ellipticity bands was suggested, although no attempt was made to assign a particular origin to any of the bands.

The rationale behind this study was to prepare derivatives of Con A in which a number of tyrosine and tryptophan residues were independently chemically modified. It was felt that the resulting CD spectra should then lend themselves to a more detailed view as to the origin of the various bands noted in native Con A. This approach has been used with considerable success in studies by Gorbunoff of several common protein systems [3,4].

## 2. Experimental

Con A was obtained from Miles-Yeda Ltd. (lot numbers 10 and 31), in 30% saturated ammonium sulphate suspension. This protein was 3 X crystallized and was used without further purification. Experimental solutions were prepared by dialyzing small aliquots of the above suspension against the preferred buffer system, usually 0.2 M NaCl, 0.02 M sodium acetate, pH 5.2. The CD measurements were carried out on a Cary model 60 recording spectropolarimeter with attached 6001 CD accessory in accordance with methodology described by Kay [1]. The procedures used for the chemical modification of tyrosine and tryptophan residues in Con A are described below.

## 3. Results and discussion

### 3.1. *Modification of tyrosine residues*

#### 3.1.1. *Reaction with tetranitromethane (TNM)*

Reaction conditions involved adding an aliquot of TNM in 95% ethanol, which represented several molar excess, to an aqueous buffered solution of the protein [5]. It was found that if the pH of the reaction mixture was much above 7.0, precipitation of the protein almost invariably occurred, e.g., at pH 8.0 even after 10 min of reaction, massive precipitation occurred. Consequently, the reaction was usually carried out overnight at pH 7.0 and this resulted in nitration of between 4 to 8 tyrosine residues per $10^5$ g of protein, as quantitated by absorbance measurements at the isobestic point (381 nm), using an extinction coefficient of 2200 $M^{-1}$ $cm^{-1}$ [5].

Fig. 1 shows a representative CD spectrum of 3-nitrotyrosyl Con A (5.2 groups modified/$10^5$ g protein) measured in acetate buffer at pH 5.2 from 250–320 nm. The band positions are essentially unchanged from those of native Con A, but the spectrum is more negative than the control, especially in the region below 280 nm.

This figure also reveals the ellipticity band generated at about 365 nm, due to the 3-nitrotyrosyl residues. Conversion of the 3-nitrotyrosyl residues into 3-aminotyrosyl ones by reduction at pH 6.8 with sodium dithionite [6] results in elimination of the 365 nm band, and the spectrum in the region of aromatic absorption more closely resembles that of native Con A.

 *North-Holland Publishing Company – Amsterdam*

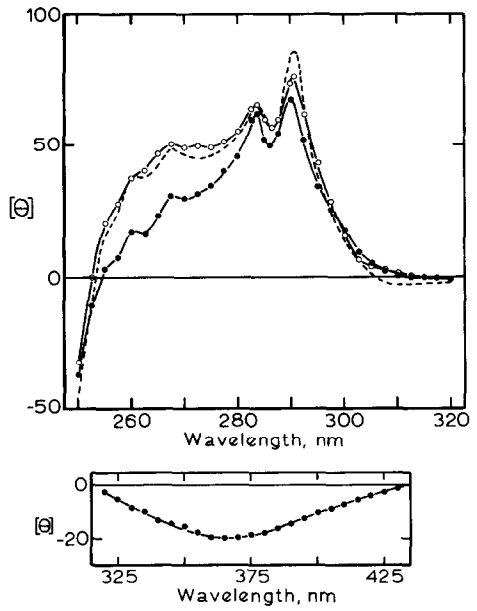


Fig. 1. Molar ellipticity values in the aromatic absorption region for native Con A (– – – –) and 3-nitrotyrosyl Con A (•–•–•) in 0.2 M NaCl, 0.02 M sodium acetate, pH 5.2. (o–o–o) represents the spectrum of 3-aminotyrosyl Con A measured in the same solvent at pH 6.8. The inset represents the CD spectrum in the visible region for 3-nitrotyrosyl Con A in acetate buffer at pH 5.2.

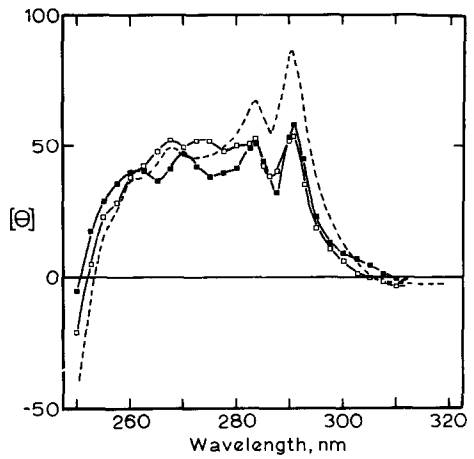


Fig. 2. Molar ellipticity values in the aromatic absorption region for native Con A (– – – –), CyF-treated Con A (▪–▪–▪) and NAI-treated Con A (□–□–□) in 0.2 M NaCl, 0.02 M sodium acetate, pH 5.2.

In a study of the CD spectrum of ribonuclease [7] it was suggested that the aromatic side chain Cotton effect noted at 278 nm in neutral pH media should be assigned to the buried tyrosyl residues which stabilize the tertiary structure of the enzyme. It is possible that a similar situation may apply with native Con A. We have found from a spectrophotometric titration study of Con A that approx. 30% of the known tyrosine residues are probably buried since they ionize with a pK value in excess of 11.5, and these abnormally ionizing residues may well be essential for maintaining the native structure. In fact Pflumm et al. [8] have alluded to this possibility by defining an irreversible transition in the CD spectrum of Con A at around pH 9.0.

As the reaction with TNM has been carried out close to pH 7, it is extremely unlikely that any of the buried tyrosine residues will be affected. If the 283 nm and the 291 nm bands are caused by the buried tyrosine or tryptophan residues then it is perhaps not surprising that this region is little affected by TNM treatment of the protein. As the 260–280 nm region is primarily altered upon nitration, then perhaps we can suggest the free tyrosines as the causative agents for this portion of the native spectrum. These bands, even in the native protein, are of lesser intensity and more poorly resolved than those at 283 nm and 291 nm. Unfortunately it has not been possible to carry out the nitration at a sufficiently high pH to react any of the abnormally ionizing tyrosines; this of course would be the ideal way to establish their precise role in the manifestation of the 283 and 291 nm bands.

It should also be mentioned that the far UV CD spectra of these nitrated samples indicated that $[\theta]_{222.5}$ drops from its value in native Con A of approximately −7,000° to about −5,500°.

3.1.2. *Reaction with cyanuric fluoride (CyF).*

The reaction of CyF with proteins is carried out in aqueous buffered solution containing 10% dioxane to keep the reagent soluble, at pH values between 9 and 12 [3,4,9]. However this particular reagent does not lend itself well to an examination of Con A, the paramount difficulty being the high pH (minimally 9) at which the reaction is carried out.

Fig. 2 shows the effect of reaction of Con A with CyF on the CD spectrum in the region of 250–320 nm.

It can be seen that although the band positions are essentially unchanged, there is an appreciable reduction in amplitude, particularly of the 283 and 291 nm bands. As will be shown later in fig. 4, these changes are accompanied by an appreciable drop in the value of $[\theta]_{222.5}$ suggesting a considerable loss of secondary structure. Additionally, the system exhibits aggregation since a sedimentation velocity run in the analytical ultracentrifuge at ∼3 mg/ml indicated a two peak system with $S_{20,w}$ values of 4 and 6 S, as compared with a value of 3.65 S for the native protein at this concentration. The CD changes produced in the region of aromatic absorption may therefore not be due to the chemical modification per se, but rather may be a reflection of the partial disruption of the molecule induced by the conditions required for reaction with CyF.

3.1.3. Reaction with *N*-acetyl imidazole (NAI).

Reaction with NAI was carried out by incubating the protein with a 60-fold molar excess of NaI in 0.01 M Tris-HCl, pH 7.5 buffer, at room temp for about 1 hr. After reaction the mixture was chromatographed on Sephadex G-25 to remove by-products which would interfere with quantitation of the treatment [10]. The modified protein was then eluted from the column in 0.2 M NaCl 0.02 M sodium acetate, and this solvent was then used for the CD measurements.

Fig. 2 illustrates a typical CD spectrum obtained for NAI-treated Con A in the aromatic region. Large changes are produced, particularly in the 283 and 291 nm bands but they are accompanied by a decrease in $[\theta]_{222.5}$ of ∼2400° from the native value, which would indicate some denaturation under these conditions (fig. 4). Thus here as with CyF, it is difficult to tell whether the changes induced are directly due to the modification of the tyrosine residues, or whether they are a consequence of disruption of the secondary structure. It should be added that since CyF and NAI react with the hydroxyl group of the tyrosine moiety rather than with the benzene ring, as is the case with TNM, it is not surprising that different types of effects are encountered.

3.2. *Modification of tryptophan residues*

3.2.1. Reaction with 2-hydroxy-5-nitrobenzyl bromide (HNBB)

Con A in 0.2 M NaCl, 0.02 M sodium acetate, pH 5.2 was reacted with HNBB ($3 \times 10^{-2}$ M) in 5% acetone and the mixture chromatographed on Sephadex G-25 using the same acetate buffer for elution [11]. The number of HNB groups coupled to the protein molecule was determined in 0.1 N NaOH from the absorbancy of these groups at 410 using an $\epsilon_{410}$ value of 18,000 $M^{-1}$ $cm^{-1}$; it was found that about 5 tryptophan residues per $10^5$ g protein had reacted.

Fig. 3 shows the CD spectrum of HNBB treated Con A in the 250–320 nm region. There appears to be a slight increase in the band area between 260 and 285 nm, but the band positions are unchanged. Additionally the 291 nm band undergoes very little alteration. Accompanying these aromatic alterations it should be noted that $[\theta]_{222.5}$ is lowered to ∼−5200° (fig. 4). Some variability was observed with this reagent in that sometimes spectra were obtained which were less positive than the control throughout the aromatic region; however in these cases $[\theta]_{222.5}$ was always much lower than the control as well.

3.2.2. Oxidation with *N*-bromosuccinimide (NBS)

Reaction with NBS may be carried out in the pH range 3–4, where hydrolytic cleavage of the polypeptide chain results, or in the range 5–7 where no scission occurs. The products of oxidation over this latter pH range is an oxindole which is relatively stable. Con A was reacted with NBS in acetate buffer at pH 5.2 with a 5–10 molar excess of reagent. Even this amount of reagent tended to cause incipient precipitation in some cases.

As fig. 3 indicates. the entire CD spectrum in the 250–320 nm range becomes slightly less positive upon NBS treatment, although the band positions are essentially unchanged. At the same time the far UV region indicates little disruption of secondary structure, $[\theta]_{222.5}$ being −6500 as compared to −7000° for the native protein (fig. 4).

3.2.3. Alkaline hydrogen peroxide-dioxane treatment

It has been found that tryptophan residues may be oxidized over a pH range of 8.1–9.4 by $H_2O_2$ in 0.5 M bicarbonate buffer containing 10% dioxane [12]. When this procedure was applied to Con A, a shift of the far UV ellipticity band to 215 nm was

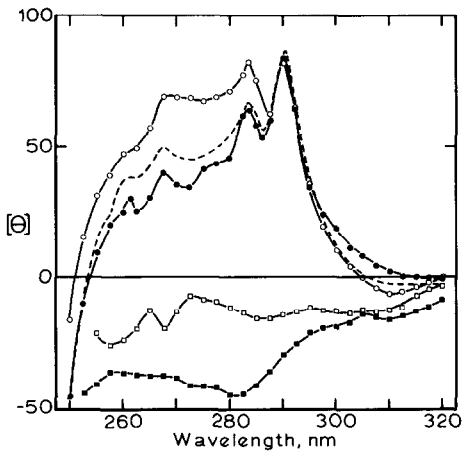


Fig. 3. Molar ellipticity values in the aromatic absorption region for native Con A (– – –), NBS-treated Con A (•–•–•), HNBB-treated Con A (o–o–o), and $H_2O_2$-treated Con A (□–□–□) in 0.2 M NaCl, 0.02 M sodium acetate, pH 5.2. (■–■–■) represents molar ellipticity values for Con A in 0.5 M bicarbonate, 10% dioxane at pH 8.1.

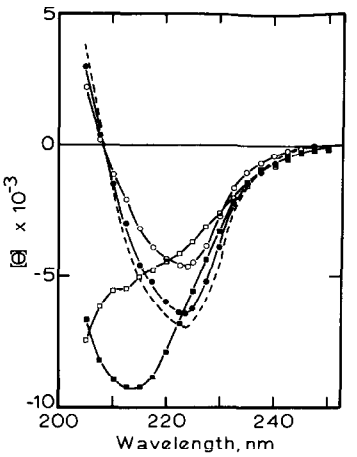


Fig. 4. Molar ellipticity values in the far ultraviolet region for native Con A (– – –), NBS-treated Con A (•–•–•), NAI-treated Con A (o–o–o) and $H_2O_2$-treated Con A (□–□–□) in 0.2 M NaCl, 0.02 M sodium acetate, pH 5.2. (■–■–■) represents molar ellipticity values for Con A in 0.5 M bicarbonate, 10% dioxane at pH 8.1.

noted (fig. 4), which suggests extensive disruption of the native $\beta$ structure and the formation of random coil. Consequently the changes produced in the region of aromatic absorption are very dramatic (fig. 3). Fig. 4 also shows the far UV spectrum of Con A in 10% dioxane-bicarbonate buffer at pH 8.1 in the absence of $H_2O_2$ where the formation of random coil is also exhibited.

## 4. Conclusions

The CD results obtained with those chemically modified samples of Con A which showed little secondary structure alteration, suggest that the various bands probably represent composite contributions from both the tyrosine and tryptophan residues. For example, the results of TNM treatment revealed a decrease in the band magnitude in the 260–280 nm region on the one hand, while HNBB treatment increased the positivity of the spectrum in the same area. One is thus led to the conclusion that interaction between closely juxtapositioned tryptophan and tyrosine residues may be responsible for the ellipticity bands in this region.

Because of the instability of Con A to extremes of pH or vigorous reaction conditions, it has not proved possible to react all of the tryptophan or tyrosine residues known to be present in the molecule. It is quite conceivable that it is these abnormal groups which are responsible for most of the optical activity in the 250–320 nm region. If this is indeed the case it will be a difficult problem unequivocally to establish the precise chromophores contributing to the various aromatic ellipticity bands.

It may be of interest in this connection to refer to the CD measurements carried out on some tryptophan derivatives at 77° K by Strickland et al. [13]. The CD spectrum of *N*-acetyl-L-tryptophanamide in tetrahydrofuran-diglyme at 77° K exhibits a marked degree of similarity to that of native Con A at pH 5.2. In particular, it is characterized by two well-resolved fine-structure positive bands at 291 and 284 nm, a third weaker positive band at 278 nm and a broad positive region between 260 and 270 nm. If one assumes that buried tryptophan residues are contributing appreciably to the aromatic optical activity in Con A, then such residues will be in a much higher state of conformational rigidity than exposed ones. In this case the situation may be analagous to that of tryptophan derivatives cooled to extremely low temperatures wherein one observes an enhancement of the CD fine structure relative to that noted at room temp.

Finally, it should be borne in mind in all these stu-

dies that the observed alterations in the aromatic CD bands might not be a direct result of chemical modification but rather an indirect one due to an alteration in environment in the vicinity of certain of the aromatic residues. The fact that alterations in the CD spectrum have been obtained by both tyrosine and tryptophan modifying reagents would perhaps lend support to this idea.

## Acknowledgements

This work was supported by research grants from the Medical Research Council of Canada (MRC-MT-1223), the Life Insurance Medical Research Fund and the Muscular Dystrophy Association of Canada.

## References

[1] C.M. Kay, FEBS Letters 9 (1970) 78.
[2] W.D. McCubbin, K. Oikawa and C.M. Kay, Biochem. Biophys. Res. Commun. 43 (1971) 666.
[3] M.J. Gorbunoff, Biochemistry 8 (1969) 2591.
[4] M.J. Gorbunoff, Arch. Biochem. Biophys. 138 (1970) 684.
[5] M. Sokolovsky, J.F. Riordan and B.L. Vallee, Biochemistry 5 (1966) 3582.
[6] M. Sokolovsky, J.F. Riordan and B.L. Vallee, Biochem. Biophys. Res. Commun. 27 (1967) 20.
[7] R.T. Simpson and B.L. Vallee, Biochemistry 5 (1966) 2531.
[8] M.N. Pflumm, J.L. Wang and G.L. Edelman, J. Biol. Chem 246 (1971) 4369.
[9] K. Kurihara, H. Horinish and K. Shibata, Biochim. Biophys. Acta 74 (1963) 678.
[10] J.F. Riordan, W.E.C. Wacker and B.L. Vallee, Biochemistry 4 (1965) 1759.
[11] H.R. Horton, and D.E. Koshland, J.Am. Chem Soc. 87 (1965) 1126.
[12] Y. Hachimori, H. Horinishi, K. Kurihara, and K. Shibata, Biochim. Biophys. Acta 93 (1964) 346.
[13] E.H. Strickland, J. Horwitz, and C. Billups, Biochemistry 8 (1969) 3205.