The Optical Rotatory Dispersion of Aromatic Amino Acids and the Side Chain-dependent Cotton Effects in Proteins*

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SUMMARY

The optical rotatory dispersion of L-tryptophan, L-tyrosine, L-phenylalanine, and some of their derivatives was investigated in the ultraviolet region as a function of solvent, pH, and temperature.

All aromatic transitions accessible to study were found to be optically active, but the rotatory strength was found to be low. The long wave length tyrosine bands at 280 nm showed a 100-fold lower rotatory strength when compared to the n - π* transition of the peptide bond.

The greatly increased rotatory strength of aromatic transitions in some proteins must thus be due to vicinal perturbations having their source in the conformation of protein.

Investigation of solvent and temperature dependence of amino acid rotation showed that only a change in the charge on the molecule produced measurable increase in the rotatory strength.

It is proposed that the enhanced aromatic Cotton effects in some proteins are produced by interaction of the aromatic residue with a neighboring charge or other polarizable group.

Since long wave length Cotton effects (250 to 350 nm) are accompanied by a system of strong Cotton effects at the low wave length transitions of the aromatic residues, the usual estimates of α-helix may be difficult or impossible in protein showing enhanced aromatic rotation.

The rotatory contribution from the aromatic side chains to the observed rotatory dispersion curve of proteins has recently received considerable attention, mostly because of the presence of unusually clear and strong Cotton effects in the aromatic absorption region of lysozyme (1), cytochrome c (2), and carbonic anhydrase (3, 4). The existence of aromatic Cotton effects in the optical rotatory dispersion of amino acids was first predicted by Strem, Krishna-Prasad, and Schellman (5) from the form of rotatory dispersion curves in the visible. The effects observed in the ultraviolet absorption bands of L-tyrosine and L-phenylalanine have been described previously (6, 7). Theoretical considerations show that the expected rotatory strength of an intrinsically symmetrical chromophore in β position to the asymmetrical carbon is small (6, 8). The amplitude of these effects is such that any substitution of the asymmetrical carbon that increases the symmetry of the surroundings as seen by the aromatic transitions or introduces new and strong Cotton effects may render the aromatic Cotton effects undetectable.

Thus, for example, in the case of acetyl-L-tyrosine amide and ester, the effects are of the magnitude that easily escape detection (7). In accordance with these findings, one can predict that the expected rotatory contribution from the aromatic side chains of the protein to the observed backbone rotation is a small one. In the case in which no additional vicinal effects are present, for example, in unfolded or so called random coil form, the effects should not be larger than for L-acetyl amides of the free amino acids, and thus at best would be just barely detectable. This agrees well with the observation that the majority of proteins in the native state and, as far as we know, all the proteins in unfolded state show a smooth ORD curve through the region of aromatic absorption.

Most interesting, of course, are the proteins that despite the above reasoning show clear-cut and strong Cotton effects in the aromatic region. Of several effects reported, those of the different forms of carbonic anhydrase are of such magnitude that the question arises whether we can at all assume that the massive Cotton effects are due to the aromatic side chains.

The rotatory strength of the electronic transitions from these groups should, in this case, be enhanced 25- to 100-fold when compared to the same transitions in amino acids and simple peptides. The nature of structural details responsible for such an increase is of great interest since it contains unique information about the conformation of the protein.

We have in the present investigation tried to establish the presence, form, and strength of Cotton effects from all experimentally accessible absorption bands of the aromatic side chains. We hope that these findings will help us to identify the contributions from different side chains to the aromatic Cotton effects encountered in proteins. We have further studied the effect of solvent, temperature, and vicinal charges on the magnitude of these Cotton effects in a search for a possible explanation for enhancement of such aromatic effects in proteins.

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1 The abbreviation used is: ORD, optical rotatory dispersion.
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Fig. 1. The ORD of L-phenylalanine in aqueous solution, pH 6.9, O—O; L-phenylalninol in aqueous solution, pH 8.9, •—•; and L-leucinol in aqueous solution, pH 10.8, □—□. The temperature on all occasions was 27°C.

METHODS AND MATERIALS

Chromatographically pure L-phenylalanine (Lot 4514), L-phenylalaninol c.p. (Lot 1519), N-acetyl-L-phenylalanine ethyl ester, L-tryptophan (analytical grade; Lot L 1555), N-acetyl-L-tryptophan ethyl ester c.p., chromatographically pure L-tyrosine (Lot 340), and N-acetyl-L-tyrosine amide were purchased from Mann. L-Tyrosinol-sulfate (Lot 0500111) was purchased from ICN Corporation. L-Leucinol (technical grade) was from Fluka A.G. (Switzerland), and L-2-benzyl amino-1-propanol (Lot B1680) was from Aldrich. The rotation was measured on a Cary model 60 spectropolarimeter. The temperature was, unless otherwise stated, 27°C ± 0.5 for all measurements. In cases in which the measured rotation is very small and noise level is high, the rotation shown represents the average of several runs with different concentrations of the solute. In case the error limits are higher than instrumental, they are indicated in drawings as the maximum deviation from the average of several runs.

The ORD measurements of L-tryptophan and its derivatives present extra difficulties. In addition to the very unfavorable ratio of rotatory strength to absorptivity, the fluorescence of the indole side chain produced, at higher concentrations, appreciable amounts of stray light. With sample absorbance higher than 2, the stray light caused erroneous readings of rotation. The resulting false Cotton effects can easily be distinguished from true rotation as they do not show linear concentration dependence. As the result of such experimental difficulties, the form of the tryptophan ORD curve around 280 mµ remains undetermined in details. The rotation due to the 291 mµ transition, on the other hand, is well characterized.

RESULTS

The rotation in ORD measurements has been expressed on molecular rotation [\( \alpha \)]

\[
[\alpha] = \frac{\alpha}{MW}
\]

where \([\alpha]\) — specific rotation and \(MW\) — molecular weight. The unit is degrees per cm per 100 m solution. The solvent field correction in form of the factor \(3/(n^2 + 2)\) commonly used in reporting the rotation of proteins and polypeptides has not been used in this investigation. In methanol solutions, such a correction would be arbitrary in the short wave length ultraviolet region since the refractive index in the vicinity of a solvent absorption band cannot be calculated from the Sellmeier equation. The difference in the absolute values of rotation between the amino acids as given in this publication and the assumed side chain contributions in proteins from the same groups contains, thus, a systematic difference of 10 to 20%.

Fig. 1 shows the ORD of L-phenylalanine and L-phenylalaninol. The dominating effect in the case of L-phenylalanine is the positive Cotton effect, characterized by a peak of 221 mµ, commonly attributed in amino acids to the \(n \rightarrow \pi^*\) carbonyl transition (9). The Cotton effect at the long wave length, aromatic absorption bands of the phenyl group in L-phenylalanine were described in a previous publication (6). Their magnitude is such that on the scale employed in Fig. 1 they are not discernible. The effects centered around 260 mµ are, of course, present in the ORD pattern of L-phenylalaninol (Fig. 2) and the N-acetyl ester of L-phenylalanine (Fig. 3). As predicted (6) in all cases, the amplitudes of these effects are very small, never more than 50°.

Fig. 2. The optical rotatory dispersion of L-phenylalaninol in the aromatic wave length region, aqueous solution, pH 8.9. Temperature was 27°C.
The rotation of \( l \)-phenylalaninol, Fig. 1, is dominated by a short wave length Cotton effect with a sharp trough at 218 mp. The wave length would allow us to ascribe it tentatively to the 210 mp transition of the phenyl ring. Of course, there is always a remote possibility that the 218 mp trough is due to a transition from the \( CH_2OH \) group shifted to higher wave length. In order to exclude such a possibility, the ORD of an amino alcohol with aliphatic side chain, \( l \)-leucinol, was recorded in the same wave length region. There is no reason to believe that the electronic transitions of the \( CH_2OH \) group in both alcohols should occur at different wave lengths, provided that the same solvent is used. Therefore, irrespective of sign and magnitude, the position of a trough of the Cotton effect due to such a transition should show up at the same wave length. \( l \)-Leucinol shows a plain rotatory dispersion curve down to 208 mp, a result supporting the assignment of the 218 mp trough to an aromatic transition. Returning to \( l \)-phenylalaninol, the positive Cotton effect with a peak of 221 mp is evidently a combination of the carbonyl effect with the aromatic Cotton effect described for \( l \)-phenylalaninol. A radical change in pH does influence the ORD of \( l \)-phenylalaninol, but the effect on the long wave length aromatic Cotton effects (Fig. 4) is moderate.

Both alcohols shown in Fig. 1 are, under the indicated experimental conditions, predominantly uncharged. The introduction of charge on the \( NH_2 \) group in case of phenylalaninol results in an increase of aromatic Cotton effects, the magnitude of which is comparable to the observed charge effects in \( l \)-phenylalaninol (Fig. 4).

The next amino acid investigated was \( l \)-tryptophan, the ORD of which in water solution is shown in Fig. 5. The precision in the detail of the long wave length Cotton effects is poor when compared to \( l \)-phenylalanine, although the rotatory strength of these transitions as judged from the amplitude is higher. This is due to the high molar absorptivity of indole transitions when compared to the benzene ring. Indole absorptivity forces us to use lower concentrations with resulting increase in error. As was the case for \( l \)-phenylalanine, the short wave length Cotton effect with a deep trough at 212 mp represents a combination of two effects, the carbonyl effect and the 215 mp aromatic transition. This explains the vastly increased magnitude of the negative trough of the combined Cotton effects when compared with the carbonyl effect in aliphatic amino acids (9).

The weak Cotton effect system of the long wave length indole absorption is retained in the \( N \)-acetyl ethyl ester of \( l \)-tryptophan, the rotation of which is shown in Fig. 6. The amplitudes of the effects have apparently diminished somewhat, as in the case of derivatives of \( l \)-phenylalanine.

In the case of \( l \)-tyrosine, the situation is somewhat more complicated since the rotatory strength of both the ionized and non-ionized forms of the phenol group may show an increase due to the availability of nonbonding electrons from oxygen (6). In case that the Cotton effects of aromatic transitions and the carbonyl Cotton effect have opposite signs, the resulting curve may very well have the form of a single effect of arbitrary amplitude. A single effect of moderate size is actually seen in case of \( l \)-tyrosinol. The amplitude and position of the effect (Fig. 7) agree well with those reported by Hooker and Tanford (7). That the apparent single Cotton effect is a superposition of several effects can easily be seen from the ORD of \( l \)-tyrosinol. Here, the absence of carbonyl groups allows us to distinguish the aromatic contribution, which in the 280 to 230 mp region consists of at least three distinguishable Cotton effects. The
The ORD of L-tryptophan in aqueous solution, pH 7.3, and temperature of 27°C.

The ORD of N-acetyl-L-tryptophan ethyl ester in methanol. Temperature was 27°C.

The ORD of L-tyrosine in aqueous solution, pH 5.0, and temperature of 27°C (○—○), and the ORD of L-tyrosinol-sulfate in aqueous solution with a temperature of 27°C (○—○).

The form of the double effect at 280 μm reminds one of the aromatic Cotton effect in the ORD of poly-L-tyrosine (10). As a result of their ascribing the whole amplitude of the observed Cotton effect to the aromatic band, previous investigators were puzzled by the vanishing of this Cotton effect in the derivatives of L-tyrosine. Judging from the amplitude of the 280 μm Cotton effect of L-tyrosinol and assuming that as in the case of L-phenylalanine the aromatic contributions to the ORD of amino acid derivatives are approximately of the same magnitude as in the case of the amino alcohol, we can expect to see in the ORD of tyrosine derivatives a broad Cotton effect of a few hundred degrees amplitude. If such an effect is superposed on a steep background, it may escape detection. Still, the aromatic Cotton effect of N-acetyl-L-tyrosine amide is quite distinct in Fig. 8 and the amplitude, as expected, is a few hundred degrees. The inset in Fig. 8 shows the aromatic transition region where the form of Cotton effects has been accentuated by a freely drawn, smooth background curve.

The lower wave length transition of tyrosine at 230 μm also shows optical activity and the appropriate Cotton effect can easily be seen in case of L-tyrosine and L-tyrosinol on Fig. 7.

In order to study the effect of solvents, temperature, and the influence of charges on the aromatic rotatory bands, we have used L-2-benzylamino-1-propanol (I) as a model substance, thereby avoiding the strong rotatory band due to carboxyl absorption. Further, the molecule can exist in a totally uncharged form or with one charge on the amino group. Likewise, the elimination of 1 CH₂ moves the benzene ring nearer to the center of asymmetry, a condition which should enhance the effect of the vicinal groups on the rotatory strength of aromatic absorption bands.

First, the ORD of alcohol (I) was studied in water, methanol, and acetonitrile. The Cotton effects in the aromatic region did not change appreciably as a function of solvent; the magnitude of the effect was, in each case, roughly equal to rotation in water solution and thus barely discernible, as seen in Fig. 9. Since the total amplitude was less than 50°, smaller changes as a function of solvent could naturally not be distinguished. The rotatory
strength of the aromatic bands in I agrees well with the corresponding effects in L-phenylalaninol.

The possible influence of solvents on the rotatory strength of aromatic transitions was further investigated with acetyl esters of L-tryptophan and L-phenylalanine. In no case could any solvent-dependent changes of magnitude be detected. The only clear solvent effect was discovered in the case of L-leucinol and, in this case, it is evidently due to transitions other than aromatic. L-Leucinol showed in water solution a plain dispersion curve with increasing negative rotation, as seen in Fig. 1. In methanol, the rotation is approximately of the same magnitude but with positive sign. This finding is in agreement with the observations of Tanford (11) on other derivatives of L-leucine. He attributed it to the influence of solvent on the peptide bond. In our case, this clearly cannot be the explanation and the change must be ascribed to polarizability of the amino group.

An attempt to influence the aromatic rotation by change of temperature in the range from 0–75° showed that the effects were relatively insensitive to the temperature. Only at the highest temperature did the amplitude of the Cotton effects seem to diminish somewhat.

The only clear-cut effect in magnitude of the aromatic rotatory strength that we could find was achieved by changing the charge on the model. Thus, when the rotation of alcohol (I) in water or methanol is compared to the rotation in 1 N HCl, it is evident that the rotatory strength has increased significantly. A 10-fold increase of the rotatory strength of the benzene bands as compared to the uncharged state is so weak that an error of 100% is very possible. The addition of methanol to the acid solution did not further influence the observed rotation.

First, it is well documented that the long wave length aromatic transitions of L-tyrosine, L-tryptophan, and L-phenylalanine, as well as those of their derivatives, are optically active although the rotatory strength of these transitions is small. Schellman and Schellman (12) have derived a simple expression for calculating the rotatory strength from the relationship between dichroism and optical rotation (13). They show that, making several simplifying assumptions such as regarding the absorption bands as Gaussian, and disregarding the change of background, the rotatory strength $R_i$ of a transition at $\lambda_i$ can be estimated from the amplitude, $[\phi]$, and width, $\Delta_i$, parameters. We assume after Schellman that

$$[\phi] = \pm 0.82 \left( [\phi]_{\text{max}} - [\phi]_{\text{min}} \right)$$

$$0.93\Delta_i = \frac{\lambda_{\text{peak}} - \lambda_{\text{trough}}}{2}$$

where $[\phi]$ is molar rotation in degrees for the peak and trough, $\lambda$ is wave length, and $\Delta$ is half-width of the absorption band expressed in arbitrary units. This gives us the rotatory strength as

$$R_i = \frac{3}{n^2 + 248N^3} \frac{h \cdot [\phi]}{\lambda_i}$$

The strongest rotation is shown by the tyrosine side chain. From the ORD curve of L-tyrosinol, we can make estimates of the necessary parameters. For this purpose, we assume that the effect is due to a single transition. This is, of course, not strictly true, as the effect seems to be a sum of at least two effects, but for an estimate of magnitude a considerable error in the width and height parameters can be allowed. In the same way, we try to estimate the strength of the 291 m$\mu$ transitions of the indole ring.
in L-tryptophan. The values calculated in this way are compared in Table I to the rotatory strength of the \( n - \pi^* \) transition of the peptide group as calculated by Holzwarth and Doty (14). One has to remember that these estimates still refer to the most favorable case with, in the case of tyrosinol, the charge on amino group and, in the case of tryptophan, with both amino and carboxyl groups charged. If the residue is an uncharged polypeptide chain, the rotational strength is certainly lower by a factor of 2 or 3.

We can compare the contributions of rotatory strength both from the side chains and the polypeptide backbone to the total measurable rotation of the protein molecule. In a protein of 300 residues, containing 10 tyrosine residues, a quite common percentage, the sum of \( R \) from the peptide bond \( n - \pi^* \) transition is of the order of \( 7000 \times 10^{-48} \), whereas the contribution from tyrosine residues is about 1 to 2 \( 10^{-48} \). This explains why, as a rule, the aromatic side chain rotation does not influence the rotation in any measurable degree. This is clearly borne out by the negligible solvent dependence of the aromatic Cotton effects in model compounds. The only exception was L-leucinol, in which case no aromatic transition was involved. The effect here is probably a secondary one with the solvent influencing the state of ionization for vicinal groups. The solvent independence of amino acid Cotton effects shows that in case of an aromatic group on \( \beta \) carbon, such secondary effects are not strong enough to influence the optical rotation.

Next, a general decrease of rotational freedom of the side chain will influence the rotational strength of the side chain chromophore only when some of the rotational positions become energetically more favorable. Here again, the interaction between the chromophore in \( \beta \) position and the other substituents of the carbon is small, the evidence in this case being the negligible temperature dependence of the aromatic contributions to the optical rotation of the model compounds. The only successful experiment resulting in an increased amplitude of the aromatic Cotton effects was the introduction of charge on L-2-benzylalanine-1-propanol, focusing our attention on the interaction of chromophores with neighboring groups.

There are thus two possible factors involved in the increase of aromatic rotatory strength. The free rotation of the side chain can be eliminated by its position in a three-dimensional structure of a macromolecule; further, the chromophore may come into close contact with other charged or polarizable groups. The positioning of such groups can very well be asymmetrical so that a new center of asymmetry is created independent of the asymmetrical carbon atom to which the side chain is attached. The probability of such assumptions is discussed in connection with protein rotation in the following paper (16).

Another question to be answered in the context of this work is whether the aromatic transitions at shorter wave length are optically active. This is more difficult to determine experimentally as all of the shorter wave length transitions overlap more or less with transitions from the carboxyl group, peptide bond, and the ester group. The clearest case is that of L-phenylalaninol, for which the trough at 218 \( \mu \) is very well situated to be due to a Cotton effect of the 210 \( \mu \) transition of the phenyl ring. The only complication is the presence of a hydroxyl group. Comparison with the ORD of L-leucinol, as discussed under "Methods and Materials," rules out the possibility of a hydroxyl contribution and the Cotton effect must be ascribed to an aromatic transition. The same is the case for the 230 \( \mu \)
transition of the tyrosine side chain, and the Cotton effect due
to it is clearly recognizable in L-tyrosinol. In the case of L-
tryptophan, the large apparent Cotton effect centered at 225
nm is so clearly asymmetrical that a superposition of the car-
boxyl effect, nicely symmetrical in the case of aliphatic amino
acids (9), with an indole transition is very probable. Conse-
quently, as far as we can judge, all aromatic transitions accessible
to direct study show optical activity. The amplitude of the
low wave length effects seems considerably larger than in the
case of the 250 to 350 nm effects. In phenylalaninol, the
210 nm effect in the charged state probably has an amplitude
of a few thousand degrees, which is about the same magnitude
as the contribution from a single peptide bond. The possibility
of distinguishing such effects in proteins is unfortunately no
better than in the case of the long wave length bands. This is
mostly due to vastly decreased precision of measurement as
compared to the 250 to 350 nm region. The best possibility
would be the 230 nm tyrosine transition that, in the case of high
tyrosine content, should be detectable when superimposed on a
relatively flat background rotation. In the case of the presence
of appreciable amounts of helix, these effects will be on sharply
rising and falling curves and probably become undetectable.
The results from the model compounds so far studied show that,
as in case of effects between 250 and 350 nm, the aromatic transi-
tions at lower wave length are difficult to detect.

The contributions from Cotton effects of this magnitude to
the Moffitt-Yang equation will be easily accommodated in the
$\delta \lambda / \lambda^2 \sim \lambda^2$ term. It is evident that a further increase in
rotatory strength of these bands will ultimately lead to change
in the value for $\lambda_c$. In cases such as carbonic anhydrase (16),
the $\lambda_c$ that somehow accommodates all these bands is 145 nm,
which quite clearly indicates that rotation from side chains has
become dominating.

This unusual strength of aromatic bands is signaled by an
approximate 50-fold increase in the rotatory strength of the 250
to 350 nm region. Consequently, an increased strength of the
long wave length Cotton aromatic effect tells us that there is a
strong possibility for a still stronger region of abnormal Cotton
effects in the shorter wave length region. Whether the possible
explanation forwarded here for amino acids is the only mechanism
for producing increased rotational strength for aromatic side
chains in proteins remains to be seen.

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