SIDE-CHAIN MOBILITY AND THE CALCULATION OF TYROSYL CIRCULAR DICHROISM OF PROTEINS

IMPLICATIONS OF A TEST WITH INSULIN AND DES-B1-PHENYLALANINE INSULIN

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ABSTRACT Previous calculations using crystal structure coordinates (Strickland and Mercola [1976], *Biochemistry.* **15:** 3857) have predicted that about 40% of the calculated tyrosyl circular dichroism of hexameric insulin is due to one of the four tyrosine residues: *viz.* the A14-tyrosine interacting with the nearby B1-phenylalanine ring group. We have tested this prediction by measuring the tyrosyl circular dichroism of an isomorphous analogue of insulin, des-B1-phenylalanine-insulin. Contrary to expectation, the resulting circular dichroism was the same as that of insulin. It is concluded that the B1-phenylalanine residue does not in fact make a large contribution to the circular dichroism of A14-tyrosine. This result is probably due to the thermal motion of the B1 and A14 ring groups not taken into account by the calculations. An example of the effects of thermal motion on the calculated circular dichroism is given and improvements that do take into account thermal motion are discussed.

INTRODUCTION

Theoretical calculations using protein crystal structures have had some success in predicting the origins of circular dichroism (CD)[1] of Soret (1, 2) and tyrosyl (3, 4) transitions of several proteins. Hsu and Woody (1) showed that the rotational strength of the Soret transition of whale myoglobin and horse hemoglobin is due to interactions

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Dedicated to Prof. Dr. Ing. Dr. h. c. mult. Helmut Zahn on the occasion of his 60th birthday (13 June 1976).

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[1] *Abbreviation used in this paper:* CD, circular dichroism, difference in extinction of left and right circularly polarized light, $\epsilon_L - \epsilon_R$, per mole per centimeter of optical path length. The quantity **u** is a vector as indicated by the boldface type.

of the heme group with nearby side-chains of aromatic amino acids. The approach was confirmed by Fleischhauer and Wollmer (2), who correctly predicted the sign inversion of the Soret-Cotton effect observed in *Chironomus* hemoglobin. Strickland (3), using the monopole distributions of Woody (5), calculated the tyrosyl CD of ribonuclease, taking into account possible interactions with the chromophores of all peptide bonds, side chains of histidine, arginine, asparagine, glutamine, aspartic acid, and glutamic acid, as well as the side chains of aromatic amino acids. Strickland and Mercola (4) extended this approach to the various aggregation states of insulin. These calculations predict the correct sign, account for about 70% of the observed CD values, and predict the fourfold increase in CD that accompanies aggregation of insulin. These methods are of potential importance for understanding the origins of tyrosyl CD, for testing structural models, and for interpreting changes in tyrosyl CD when this group serves as a probe.

As in the case of the heme transitions, the most important sources of tyrosyl CD are interactions with nearby side chains of aromatic amino acids. For example, in insulin (4) the calculations suggest that the largest source of tyrosyl CD is the interaction of A14-tyrosine with the ring of B1-phenylalanine that leads to about 40% of the total calculated tyrosyl CD of the insulin hexamer. This situation can be tested by examining sequences of insulin with alterations at the A14 or B1 positions. Here we describe a comparison of the tyrosyl CD of native insulin with an analogue of insulin that lacks the terminal B1-phenylalanine residue, des-Phe^{B1}-insulin (6). Des-Phe^{B1}-insulin is fully biologically active (6, 7), retains normal insulin receptor binding activity (8; P. Freychet, personal communication), is immunologically indistinguishable from insulin (7), exhibits "negative cooperativity" as does insulin (10),[2] has a far-ultraviolet CD spectrum extremely similar to that of insulin (7), forms rhombohedral crystals (6, 7) and undergoes the same two-zinc to four-zinc transformation as insulin (G. Bentley, personal communication). Further X-ray crystal photographs show that the des-Phe^{B1}-insulin crystals are closely isomorphous to native insulin (G. Dodson, unpublished results). Therefore it seems reasonable to expect that this derivative would be structurally identical to insulin but would have about 40% reduced tyrosyl CD owing to the absence of the B1-phenylalanine contribution. This difference could not escape observation.

Unexpectedly, the results show that the near-ultraviolet CD of insulin and des-Phe^{B1}-insulin are nearly identical. From this we conclude that the B1-phenylalanine group does not in fact contribute to the tyrosyl CD of insulin. The result is discussed in terms of enhanced thermal motion associated with the A14 and B1 ring groups, as suggested by the crystal structure refinement results (E. Dodson, G. Dodson, D. C. Hodgkin, and M. Vijayan, manuscript in preparation). An example of the effects of

[2] "Negative cooperativity" is a term used to describe the phenomenon in certain bioassays that the affinity of insulin receptors for insulin is not fixed but decreases as the occupancy of the receptors increases (e.g. DeMeyts, P., R. A. Bianco, and J. Roth. 1976. *J. Biol. Chem.* **251:** 1877–1888). For examples of the receptor binding assay see refs. 8–10.

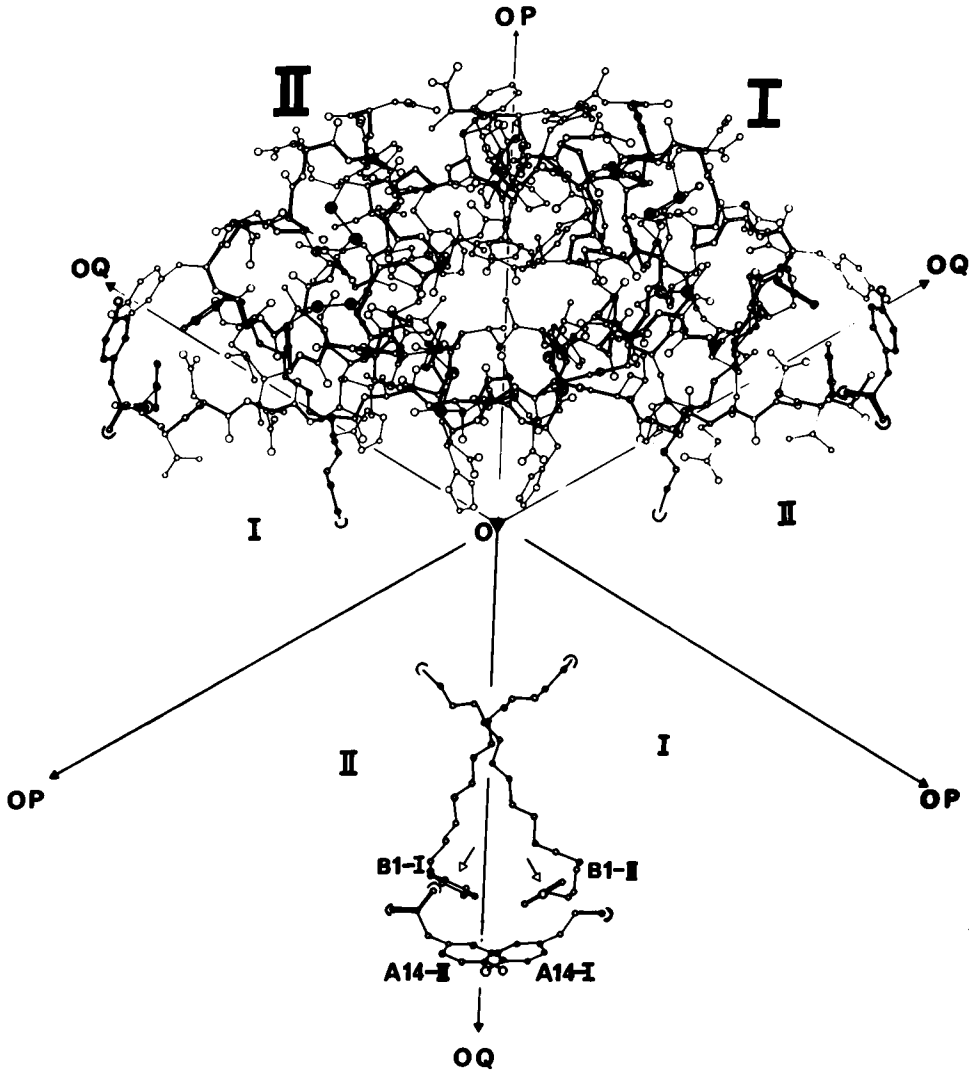

FIGURE 1 Illustration of the origin of close contacts between the 14-tyrosyl and B1-phenylalanine residues due to intra- and intermolecular contacts. The hexamer of insulin (i.e. two-zinc insulin) is composed of three dimers related by a threefold axis, ▲ (one complete dimer is shown here, top, viewed down the threefold crystallographic axis). Each dimer is composed of two similar molecules, monomers I and II, related by the noncrystallographic twofold axes, OP. As a consequence of the threefold axis, each dimer is related to adjacent dimers by the noncrystallographic axes, OQ. The contacts and molecular origin for the A14 and B1 ring groups are shown in isolation along the bottom OQ axis and in partial isolation for the other two identical OQ axes. Arrows (bottom) show the position of the B1-phenyl rings, viewed edge-on here.

thermal motion on the expected CD values is given and possible improvements to the calculations that account for the effects of thermal motion are discussed.

METHODS AND MATERIALS

Details of the preparation of des-Phe^{B1}-insulin have been described (6). The calculations of the near-ultraviolet CD of insulin have been described (4) and those for des-Phe^{B1}-insulin were calculated by the same procedures. The total calculated CD for a given aggregation state (cf. Fig. 1) is the sum of the CD of all tyrosine residues. The contributions to the CD of each tyrosine residue is calculated for the side chains of all peptide bonds and for the side chains of all histidine, arginine, glutamate, aspartate, glutamine, asparagine, and phenylalanine residues, as well as all other tyrosine residues. The calculations are carried out for each monomer, the dimer, and the hexamer of insulin (Fig. 1). Except where noted, all calculations are based on the crystal structure of rhombohedral two-zinc insulin refined to an R value of 37.6% (4). The CD measurements described here were made according to methods previously described (11).

RESULTS

The structural organization in crystalline insulin, including the important relationships between the A14-tyrosine and B1-phenyl-alanine ring groups, is illustrated in Fig. 1. The hexamer of insulin is composed of three identical dimers, each made up of two similar but not identical monomer structures: monomers I and II. Therefore the calculated (4) contributions of monomers I and II to the tyrosyl CD are different (Table I). For each of these monomers there are two major sources of calculated CD for the A14 tyrosyl groups. One source is due to intermolecular contacts, while the second source is due to intramolecular contacts. Consider intramolecular contacts first: within each monomer the A14-tyrosyl groups are less than 6 Å from their respec-

TABLE I

CALCULATED TYROSYL (275 nm) CIRCULAR DICHROISM ($\Delta\epsilon$, $M^{-1} \times cm^{-1}$) FOR INSULIN AND DES-B1-PHENYLALANINE-INSULIN

Environment	Tyr A14/phe B1,* Ring-ring interaction only	Insulin	Des-Phe^{B1}-insulin	Insulin with B1-phenyl group rotation‡
Monomer				
Monomer I	0.0	−0.39	−0.48	−0.52
Monomer II	−1.42	−0.49	+0.93	+0.50
Average	−0.71	−0.44	+0.23	−0.01
Dimer	No change	−2.08	−1.41	−1.64
Hexamer	−1.66	−3.36	−1.91	−2.32

$\Delta\epsilon$ is given per mole of monomer (6,000). For des-Phe^{B1}-insulin the absence of the phenyl ring as well as of the B1–B2 peptide bond is taken into account.

*Contribution to the B1-phenyl ring only to the $\Delta\epsilon$ of tyrosine A14.

‡The contribution of B1-phenylalanine in the crystallographically defined position was replaced by that contributed by the B1-phenyl group when in constant free rotation about the C_β–C_γ, bond as estimated by averaging for 10 equal increments of rotation about C_β–C_γ.

tive B1-phenyl groups (Fig. 1). For monomer I this proximity does not lead to significant CD. However, for monomer II, the A14-tyrosine/B1-phenylalanine interaction leads to a large negative contribution of $-1.42\ M^{-1} \times cm^{-1}$, about 43% of the total value calculated for the hexamer. All other interactions leading to CD of the A14-tyrosine of monomer II are small and tend to cancel each other.

It is emphasized that these calculations apply to the individual monomers assuming the structures found in the two-zinc hexamer of insulin. No additional contributions to the CD of either A14 tyrosine arise when the monomers are placed in the dimer environment because both A14 groups are on surfaces of the molecules far from the region of monomer/monomer contacts (Fig. 1).

The second major source of CD of the A14-tyrosine groups is due to dimer/dimer contacts in the hexamer (Fig. 1, OQ axis). There are two A14/B1 pairs on the surface of each dimer and both of these pairs are brought close to an A14/B1 pair of a neighboring dimer by the hexamer symmetry. Therefore in the hexameric state each A14-tyrosine is within 6 Å of the B1-phenyl group of the neighboring dimer and within 4 Å of the neighboring A14-tyrosine. For the A14-tyrosine of monomer I the most important additional contribution arising from hexamer formation is due to the nearby ring of A14-tyrosine of monomer II ($-0.59\ M^{-1} \times cm^{-1}$). The contribution from the B1-phenylalanine of monomer II is small. However, for the A14-tyrosine of monomer II the contribution from the neighboring B1-phenylalanine of monomer I is impressive: $-1.65\ M^{-1} \times cm^{-1}$. This is reinforced by a contribution of $-0.4\ M^{-1} \times cm^{-1}$ due to the A14-tyrosine of monomer I.

As a result of all these contributions, the A14-tyrosyl groups account for about 55% of the total calculated CD of the hexamer. Clearly the major sources of this calculated optical activity are interactions (intermolecular) with the B1-phenyl groups, which amounts to about 80% of the tyrosyl CD arising from dimer/dimer contacts or over 40% of the total calculated CD of the hexamer.

The expected consequences due to the removal of the B1-phenylalanine residue for each aggregation state are summarized in Table I. The first column gives the calculated contribution of the B1-phenyl ring to the CD of the A14-tyrosyl group of each monomer. These values largely account for the calculated differences between insulin and des-Phe^{B1}-insulin (Table I, column 3 less column 2). For the hexamer, the expected CD value for des-Phe^{B1}-insulin is reduced by more than 40% compared to that of insulin. For the dimer, the expected CD value for either insulin or des-Phe^{B1}-insulin is smaller due to lost intermolecular contacts, and the differences in CD between insulin and des-Phe^{B1}-insulin are also less, about 30%. Similarly the CD values for the monomers are expected to be further reduced (Table I).

The observed CD values of insulin and des-Phe^{B1}-insulin are summarized in Table II. At the highest concentration studied and in the presence of Zn^{2+} ions (two atoms of Zn^{2+} per six molecules of insulin), insulin is expected to be in a completely hexameric state (4, 11–13). The observed CD value of $-4.22\ M^{-1} \times cm^{-1}$ for insulin (Table II) corresponds well with an average of $-4.30\ M^{-1} \times cm^{-1}$ taken from the literature (14–16) for similar conditions well known to favor the hexameric form (4, 11–13). In

TABLE II

OBSERVED NEAR-ULTRAVIOLET CIRCULAR DICHROISM ($\Delta\epsilon$, $M^{-1} \times cm^{-1}$) FOR INSULIN AND DES-B1-PHENYLALANINE INSULIN

Molar concentration	Zinc-free		Two zinc atoms per hexamer	
	Insulin	Des-Phe^{B1}-insulin	Insulin	Des-Phe^{B1}-insulin
3.5×10^{-4}	−3.12	−3.00	−4.22	−4.22
3.5×10^{-5}	−2.68	−2.78	−3.80	−4.06
3.5×10^{-6}	−1.87	−2.07	−2.12	−2.84

In all cases $\Delta\epsilon$ given per mole of monomer (6,000) at 275 nm. Insulin and des-Phe^{B1}-insulin were measured in 0.025 M Tris-HCl buffer pH 7.8 with a Cary model 61 CD spectrometer (Varian Associates, Palo Alto, Calif.) as previously described (11). Values for insulin are taken from Wood et al. (11).

the absence of Zn^{2+} ions and at progressively lower insulin concentrations, the observed CD values decrease in agreement with the expected dissociation of insulin to dimers and monomers (4, 11–13). These (Table I) and similar measurements for solutions of insulin in mixed association states have been used in combination with the association equilibrium constants of Goldman and Carpenter (17) to estimate the "corrected observed" CD value for the dimers and monomers (4, 11). Corrected CD values for the dimer in the range of −3.0 to −3.3 $M^{-1} \times cm^{-1}$ have been obtained (4, 11). Thus from the data in Table II it appears that considerable dissociation to dimers and some monomers has occurred as a result of dilution.

The observed CD values for des-Phe^{B1}-insulin measured under identical conditions as for insulin are also shown in Table II. In striking contrast to the expected 40% reduction in the CD value of the hexameric state, des-Phe^{B1}-insulin has the same value as native insulin, −4.22 $M^{-1} \times cm^{-1}$. In the absence of zinc the observed CD value of native insulin (−3.12 $M^{-1} \times cm^{-1}$) is very similar to the CD values for a population of dimeric insulin (4, 11). The observed CD value for des-Phe^{B1}-insulin, −3.00 $M^{-1} \times cm^{-1}$, under the same conditions is also very nearly identical to that of insulin. Similarly, at lower concentrations in the absence of zinc, the CD intensities of both des-Phe^{B1}-insulin and insulin decrease to similar values. In fact at the lowest concentration in the presence of zinc, the CD intensity of des-Phe^{B1}-insulin is rather larger than that of insulin. Therefore it is apparent from these comparisons that the removal of the B1-phenylalanine residue does not lead to a decrease in the tyrosyl CD intensity of insulin.

DISCUSSION

In general there are two possible reasons for this discrepancy between theory and experiment. The crystal structure model used for the calculations may somehow be inappropriate and/or there may be an inadequacy in the method of calculation. It seems unlikely that the crystal structure is inappropriate due to differences in the structure of insulin in the crystalline and aqueous phases. A large amount of evidence from physical, chemical, biological, and immunochemical studies has been accumu-

lated about the structure in solution and has been reviewed recently (12, 13). From this it is reasonably certain that the zinc-containing hexamer of insulin in solution is essentially the same as that in rhombohedral crystals (18). Less is known about the structure of monomers and dimers of insulin in solution, though the weight of evidence favors similarity (12, 13). For example, the distance between spectroscopic probes covalently bound to terminal residues of the monomer in solution are in agreement with the crystal structure (19). In addition the asymmetry in the dimer in rhombohedral crystals has also been observed in solution (20).

Similar questions arise when considering the structure of des-Phe^{B1}-insulin, i.e. is the structure of des-Phe^{B1}-insulin, especially with respect to the A14-tyrosine, identical to that of insulin apart from the missing terminal residue? Des-Phe^{B1}-insulin is probably the most extensively characterized chemical modification of insulin known. All reported biological, immunological, and physical properties are very similar if not identical to that of insulin (6–11), suggesting very similar or identical structures. Several observations from the crystal structure analysis support this conclusion. An independent structure determination of a second form of rhombohedral insulin, four-zinc insulin, has recently been described (21). A hexamer is also present but the NH_2-terminal B-chain (B1 Phe to B8 Gly) of one monomer in each dimer (monomer I) has undergone an extended chain-to-helix transition so that the close contacts between the B1-I phenyl group and the pair of A14 phenolic groups (cf. Fig. 1) are lost and the separation is now 25 Å. As a result, the neighboring B1-II phenylalanine residue is also somewhat rearranged, leading to an increased separation of over 10 Å from the A14 phenolic pair. However, in spite of these large motions, the positions of the A14 tyrosine residues are very nearly unchanged. This is likely due to an intermolecular hydrogen bond between the hydroxyl functions of the phenolic groups and other close dimer/dimer contacts. These observations show that the position of the A14 phenolic groups in the hexamer are not dependent on the B1 residues and that the absence of these phenyl groups does not necessarily lead to changes at the A14 residues. The examination of crystalline des-Phe^{B1}-insulin also supports this view. Des-Phe^{B1}-insulin crystallizes in the same space group as insulin, and X-ray photographs to a resolution of 2.15 Å spacing show that insulin and des-Phe^{B1}-insulin are closely isomorphous. These observations suggest that it is reasonable to assume that the two-zinc crystal structure of insulin is a valid model for des-Phe^{B1}-insulin. In addition, the crystal structure has proved useful in predicting the effects of aggregation on the observed CD. For these reasons and the above, it is assumed in what follows that the crystal coordinates are a valid model for insulin and des-Phe^{B1}-insulin (see Methods and Materials).

Alternatively it is possible to identify a limitation in the calculations. The method of calculation predicts the total tyrosyl CD intensity as a function of a point monopole distribution. The fixed nature of the monopoles is due to the use of point atoms fixed by the crystal structure coordinates. This approach leads to calculations of tyrosyl CD at 0°K. Therefore comparison of calculated values to observations made at elevated temperatures may require special precautions. For example, the effects of

thermal excitation may cause the temperature dependence of tyrosyl CD, as observed for some proteins over the range of 77–298°K. For ribonuclease S and ribonuclease A the CD intensity decreases upon increasing the temperature from 77°K to 298°K (22), whereas the CD intensity of tropomyosin (J. Horwitz and B. Bullard, personal communication) is nearly independent of temperature over this range. Preliminary studies of insulin suggest that some increase in CD does occur upon cooling to 77°K (E. H. Strickland, personal communication; H. Schnitz, personal communication).

Where there is a temperature dependence of the tyrosyl CD, if the effects of thermal excitation were equal and isotropic for all atoms, it might be expected that all calculated and observed contributions to the CD intensity would be related by a simple multiplicative factor. However, for proteins it is well known from crystal structure studies at ambient temperatures that surface residues are less well stabilized than internal residues. Therefore it may be anticipated that surface ring groups may be particularly susceptible to thermal excitation. Recent nuclear magnetic resonance studies of proteins in solution at ambient temperature indeed suggest that this is the case. Some of the ring groups of the tyrosyl and phenylalanine residues in lysozyme (22), bacterial ferredoxin (23), pancreatic trypsin inhibitor (24, 25), horse ferricytochrome *c* (26), parvalbumin (27), and troponin-C (28) have been shown to rotate (22, 25, 26) or flip (22, 26) with frequencies of 10^4 s^{-1}. A second class of ring groups in these proteins is not involved in such averaging motions. The two classes of ring groups are attributed to differences in the thermal stability of surface and internal residues (22, 23, 25–26).

In the case of insulin, surface ring groups such as the A14-tyrosine and B1-phenylalanine residues are likely candidates for such motions in solution. As an illustration, we have calculated the effects of rotation on the total calculated CD intensity by assuming free rotation of the B1-phenylalanine ring group about the C_β–C_γ bond, and the results are given in Table I. When rotation of the B1-phenyl ring is included, the CD values of insulin calculated for each association state are reduced and much closer to those calculated for des-Phe^{B1}-insulin. This result suggests that the A14/B1 pair may not generate as much CD as has been calculated for native insulin if rotation occurs for one or both of these ring groups, and that then the decrease expected for des-Phe^{B1}-insulin would have been overestimated. In general the reduction in CD is not surprising. Although incremental rotations may lead to some positions with large CD contributions, when averaged over a complete rotation, positive and negative contributions would tend to cancel. A similar effect then may be expected for the A14-tyrosine residue. In agreement with these conclusions, the current work on the crystallographic refinement of the rhombohedral crystal structure suggests that these two ring groups do have above average thermal parameters (E. Dodson, G. Dodson, D. C. Hodgkin, M. Vijayan; manuscript in preparation).

The illustration using rotation, one plausible mode of thermal motion, is a particular example and so does not provide a general basis for correcting the calculated CD values. However, in crystal structure refinement the thermal properties of atoms can be determined and are most easily described in terms of the temperature factor B (30).

For proteins, thermal motion may be approximated by isotropic B values related to the rms displacement, **u**, due to vibration or other modes by $B = 8\pi^2\overline{u^2}$ (30). Thus the B values are time-averaged descriptions of atomic motion. For internal residues of proteins these values are commonly in the range 5–15 Å^2, whereas for surface groups these values may be considerably higher. The recent crystallographic refinement results for insulin show that the average atomic B values for the ring group of the A14-tyrosine and B1-phenylalanine are about 25 Å^2 and 35 Å^2, respectively, i.e. somewhat elevated. The possible consequences of these values for the calculated tyrosyl CD intensity may be seen by noting that, for isotropic B values, atomic displacements are equal in all directions and $\bar{\mathbf{u}} = 0$. Then the standard deviation of **u**, $\sigma(u)$, is simply $\sqrt{\overline{u^2}} = \sqrt{B/8\pi^2}$. The standard deviation, in turn, defines the spread or width of a normal distribution of displacement about a mean position for a vibrating atom or any other quantity rigidly fixed to the atom such as a monopole. The important quantity for calculation of the CD influenced by these considerations is the interaction potential between any two monopoles (1, 3). For large B values and small monopole separations the normal distribution function may show that the two monopole distributions overlap. It is clear from simple electrostatics that in the case of overlapping charge distributions the calculated potential and resulting CD will be decreased. For ease of discussion here we define an equivalent radius, r_e, of a vibrating monopole as the radius of a sphere containing over 99.7% of the monopole charge, i.e. $r_e = 3 \cdot \sigma(u)$. Then two monopoles whose Σr_e is less than their center-to-center separation (as defined by the crystal structure) will not overlap and will have an interaction potential and CD equal to that of point atoms. For example, for two average monopoles with $B \sim 10$ Å^2, overlap does not occur for separations over 2.0 Å. Separations of more than 2.0 Å are in fact the case for over 90% of all the important monopole interactions calculated for insulin (4). Therefore calculations with average or well-ordered atoms at 298°K are likely to lead to a similar result as that for 0°K. However, if one or both of the interacting groups are disordered, say $B_1 = 35$ Å^2 and $B_2 = 25$ Å^2, (as might be expected for the B1 and A14 ring atoms), then Σr_e is 3.8 Å or considerably larger than, for example, the average minimum separation of monopoles in any of the four A14/B1 pairs in insulin, $\bar{r}_{min} \simeq 2.0$ Å. Therefore in this case an overlap in some of the monopole pairs and a reduction of the interaction potential must be expected. Thus the preliminary vibrational parameters of crystalline insulin, as studied at ambient temperature, predict that the observed CD of the A14-tyrosine/B1-phenylalanine interactions may be less than that calculated at 0°K. This is consistent with the model calculation, where the B1-phenyl group was allowed to rotate freely. These considerations, then, provide a qualitative explanation why removal of B1-phenylalanine had no effect on the tyrosyl CD of insulin.

The line of reasoning developed here suggests several possible ways to quantitatively calculate the tyrosyl CD of proteins at ambient temperature. In the simplest case each fixed monopole would be replaced by a Gaussian charge distribution centered at the monopole coordinate with an integrated volume equal to that of the monopole and

with standard error of $\sqrt{B/8\pi^2}$. In this way monopoles with large B values become symmetrically "smeared" in space due to thermal excitation while monopoles with small B values approximate point monopoles as before. There are several other possible advantages in this approach. For one, B values represent the observed effects of all disordering influences for each atom (or monopoles) and so are independent of any particular model, such as partial or free rotation, vibration, or other modes. For another, the use of a Gaussian distribution of positions means that all "allowed" local variations in the structure, which considered separately may produce significantly differing $\Delta\epsilon$ values, may now all enter into the calculations weighted by their probabilities of occurrence. Previous results have in fact shown that small changes in the coordinates led to significantly different $\Delta\epsilon$ values even though the total CD was not much affected by the changes (4). The methods discussed here may minimize this sensitivity, and small changes in the coordinates during the refinement process will not necessarily lead to large changes in $\Delta\epsilon$ values. However, it remains to be shown that this treatment when combined with the final crystallographic coordinates and B values will lead to a quantitative explanation of the results observed with des-Phe^{B1}-insulin. These studies are in progress.

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REFERENCES

1. HSU, M. C., and R. W. WOODY. 1971. The origin of the heme cotton effects in myoglobin and hemoglobin. *J. Am. Chem. Soc.* **93**:3515–3525.
2. FLEISCHHAUER, J., and A. WOLLMER. 1970. The influence of the aromatic amino acid side-chains on the sign of the Soret cotton effect in *Chironomas* hemoglobin. *Z. Naturforsch. Teil B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol.* **27B.1**:530–532.
3. STRICKLAND, E. H. 1972. Interactions contributing to the tyrosyl circular dichroism bands of ribonuclease S and A. *Biochemistry.* **11**:3465–3474.
4. STRICKLAND, E. H., and D. A. MERCOLA. 1976. Near ultraviolet tyrosyl circular dichroism of pig insulin monomers, dimers and hexamer. Dipole-dipole coupling calculations in the monopole approximation. *Biochemistry.* **15**:3875–3883.
5. WOODY, R. W. 1968. Improved calculation of the $n\pi^*$ rotational strength in polypeptides. *J. Chem. Phys.* **49**:4797–4806.
6. BRANDENBURG, D. 1969. Des-Phe^{B1}-Insulin, ein kristalles Analogon des Rinderinsulins. *Hoppe-Seyler's Z. Physiol. Chem.* **350**:741–750.
7. BRANDENBURG, D., H.-G. GATTNER, M. WEINERT, L. HERBERTZ, H. ZAHN, and A. WOLLMER. 1970. Structure function studies with derivatives and analogs of insulin and its chains. *Excerpta Med. Int. Cong. Ser.* **231**:363–375.
8. GLIEMAN, J., and S. GAMMELTOFT. 1974. The biological activity and the binding affinity of modified insulins determined on isolated fat cells. *Diabetalogia.* **10**:150–163.
9. FREYCHET, P., D. BRANDENBURG, and A. WOLLMER. 1974. Receptor binding assay of chemically modified insulins. *Diabetalogia.* **10**:1–5.

10. DE MEYTS, P. 1977. *In* Methods in Receptor Research. M. Blecher, editor. M. Dekker A.G., Basel, Switzerland. Volume 9. In press.
11. WOOD, S., T. L. BLUNDELL, A. WOLLMER, N. LAZARUS, and R. NEVILLE. 1975. The relation of conformation and association of insulin to receptor binding; X-ray and circular dichroism studies on bovine and hystricomorph insulins. *Eur. J. Biochem.* **55**:531–543.
12. HODGKIN, D. C., and D. A. MERCOLA. 1972. The secondary and tertiary structure of insulin. *Handb. Physiol. Endocrinol.;* Section 7, (Endocrinology, Vol. I), Chapter 8. 139–157.
13. BLUNDELL, T. L., G. DODSON, D. C. HODGKIN, and D. A. MERCOLA. 1972. Insulin: the structure in the crystal and its reflections in chemistry and biology. *Adv. Protein Chem.* **26**:279–402.
14. MORRIS, J. W. S., D. A. MERCOLA, and E. R. ARQUILLA. 1968. An analysis of the near ultraviolet circular dichroism of insulin. *Biochim. Biophys. Acta.* **160**:145–150.
15. MENENDEZ, C., and T. HERSKOVITS. 1970. Optical rotatory dispersion and circular dichroism studies on insulin and its trypsin-modified derivative. *Arch. Biochem. Biophys.* **140**:286–294.
16. ETTINGER, H. J., and S. N. TIMASHEFF. 1971. Optical activity of insulin. I. On the nature of the circular dichroism bands. *Biochemistry.* **10**:824–840.
17. GOLDMAN, J., and F. C. CARPENTER. 1974. Zinc binding, circular dichroism and equilibrium sedimentation studies on insulin (bovine) and several of its derivatives. *Biochemistry.* **13**:4566–4574.
18. BLUNDELL, T. L., J. F. CUTFIELD, S. M. CUTFIELD, E. J. DODSON, G. G. DODSON, D. C. HODGKIN, D. A. MERCOLA, and M. VIJAYAN. 1971. Atomic positions in rhombohedral 2-zinc insulin crystals. *Nature (Lond.).* **231**:506–511.
19. MERCOLA, D. A., J. W. S. MORRIS, and E. R. ARQUILLA. 1972. Use of resonance interaction in the study of chain folding of insulin in solution. *Biochemistry.* **11**:3860–3874.
20. LED, T. J., D. M. GRANT, W. J. HORTON, F. SUNDBY, and K. VILEHELMAN. 1975. Carbon-13 magnetic resonance study of structural and dynamical features in carbamylated insulins. *J. Am. Chem. Soc.* **97**:5997–6008.
21. BENTLEY, G., E. DODSON, G. DODSON, D. C. HODGKIN, and D. A. MERCOLA. 1975. The structure of insulin in 4-zinc insulin. *Nature (Lond.).* **261**:166–168.
22. STRICKLAND, E. H. 1974. Aromatic contributions to circular dichroism spectra of proteins. *Crit. Rev. Biochem.* **2**:113–175.
23. CAMPBELL, I. D., C. M. DOBSON, and R. J. P. WILLIAMS. 1975. Nuclear magnetic resonance studies on the structure of lysozyme in solution. *Proc. R. Soc. Lond. A. Math. Phys. Sci.* **345**:41–59.
24. DOBSON, C. M., G. R. MOORE, and R. J. P. WILLIAMS. 1975. Assignment of aromatic amino acid PMR resonances of horse ferricytochrome C. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **51**:60–65.
25. PACKER, E. L., H. STERNLICHT, and J. C. RABINOWITZ. 1972. The possible role of aromatic residues of *Clostridium acidiurici* ferredoxin in electron transport. *Proc. Natl. Acad. Sci. U.S.A.* **69**:3278–3282.
26. KARPLUS, S., G. H. SNYDER, and B. H. SYKES. 1973. A nuclear magnetic resonance study of bovine pancreatic trypsin inhibitor. Tyrosine titrations and backbone NH groups. *Biochemistry.* **12**:1323–1335.
27. WÜTHRICH, K., and G. WAGNER. 1977. NMR investigation of the dynamics of the aromatic amino acid residues in the basic pancreatic trypsin inhibitor. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* In press.
28. CAVE, A., J. PARELLO, C. M. DOBSON, R. J. P. WILLIAMS. 1976. Conformational mobility within the structure of muscular parvalbumins. An NMR study of the aromatic resonances of phenylalanine residues. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **65**:190–195.
29. LEVINE, B., D. MERCOLA, J. THORNTON, and D. COFFMAN. 1977. Calcium binding by troponin-C. A proton magnetic resonance study. *J. Mol. Biol.* In press.
30. JAMES, R. W. 1967. The optical principle of the diffraction of X-rays, *In* The Crystalline State. L. Bragg, editor. Bell & Sons, Ltd., London, England.