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Crystal Structure of a Sweet Protein, Monellin, at 5.5-Å Resolution†

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ABSTRACT: A 5.5-Å resolution crystal structure of an intensely sweet protein, monellin, has been determined on the basis of four isomorphous heavy-atom derivatives. The structure reveals many protruded features unlike most globular proteins. There are four monellin molecules in the asymmetric unit of the crystal and two dimer molecules related by a noncrystallographic twofold axis.

Monellin, isolated from *Dioscoreophyllum cumensii*, a tropic berry also called the serendipity berry, is a protein which tastes extremely sweet (van der Wel, 1972; Morris & Cagan, 1972). The sweet sensation elicited by monellin is roughly 100 000 times as intense as that of sucrose when compared on a molar basis and 3000 times as intense when compared on a milligram basis. Monellin has excellent potential for being used as a natural substitute sweetener.

The only other protein known to taste sweet is thaumatin, which is also derived from an African berry. Although monellin and thaumatin display little sequence homology (Frank & Zuber, 1976; Iyengar et al., 1978), immunological studies show cross-reactivity (van der Wel & Bel, 1978; Hough & Edwardson, 1978), suggesting that the two sweet proteins may indeed share common structural features which may be involved in the sweet taste phenomenon. Furthermore, one of these studies (Hough & Edwardson, 1978) shows that several other sweet compounds, such as saccharin, L-aspartyl-L-phenylalanyl methyl ester, sucrose derivatives, cyclamates, and neohesperidin dihydrochalcone, compete for the antibody raised against thaumatin. This suggests that these compounds may have structural features common, in parts, with thaumatin.

Monellin is a small protein with a molecular weight of 10 700. It contains no carbohydrate. The molecule is composed of two nonidentical peptide chains (Bohak & Li, 1976), and the tertiary structure of the molecule is essential for the taste effect to take place as demonstrated by circular dichroism studies using a variety of denaturing conditions (Jirgensons, 1976).

Large well-shaped crystals were obtained from a solution containing 4 mg/mL monellin (purchased from Worthington Biochemical Corp., Freehold, NJ), 15% (w/w) polyethylene glycol (average molecular weight of 6000), and 10 mM phosphate buffer at pH 7.2 and equilibrated with 33.3% (w/w) polyethylene glycol at 4 °C by a vapor diffusion method (Tomlinson & Kim, 1981). Monellin crystallizes in a space group $P2_1$ with cell parameters of 39.1 × 71.5 × 86.9 Å^3 with $\beta = 107.6°$. The density of the crystals (1.88/cm^3) was determined by using a density gradient formed by xylene and carbon tetrachloride and calibrated with a series of CsCl solutions. Partial specific volume of monellin calculated from the amino acid composition is 0.73 cm^3/g. Molecular weight of an asymmetric unit of the unit cell calculated by using the above values is 46 000, corresponding to about four monellin molecules consisting of eight peptides. This gives V_m, the ratio of the volume per unit molecular weight, to be 2.7 Å^3/dalton, which is normal for protein crystals. Still photographs show that the crystals diffract better than 2.3-Å resolution.

Experimental Procedures

X-ray diffraction data have been collected for four heavy-atom derivatives by using an ω scan mode on a four-circle automatic diffractometer at 4 °C. The heavy-atom compounds used were $KAuCl_4$, K_2PtCl_4, $HgCl_2$, and $Pd(NH_3)_2Cl_2$. Buffered solution of each compound was introduced into monellin crystals in mother liquor at a concentration of 0.5–1.0 mM and left for 1–60 days before data collection.

Phase refinement for the protein data was carried out by the method of multiple isomorphous replacement (Blow & Crick, 1959; Dickerson et al., 1961). During the first five cycles of the least-squares refinement, occupancies were held constant while positional parameters and temperature factors were varied. During the next five cycles, temperature factors were held constant and the positional parameters and occupancies were varied. The final positions, number of sites, R modulus

$$\Sigma||F_{PH} - |F_P + f_H||/\Sigma f_H$$

and weighted R modulus are listed in Table I.

In calculating the electron density map at 5.5-Å resolution, in order to minimize any rippling effect due to termination of Fourier series at 5.5 Å, an artificial temperature factor of 300 Å^2 was applied. With this temperature factor the structure factor amplitudes at 5.5-Å resolution are reduced to about 9% of its original value, thus effectively eliminating any series termination errors. This approach significantly improved the background noise level of the electron density map without noticeably altering electron density within the molecular envelope, thus facilitating identification of the molecular boundary.

To search for possible local rotation axes using a method developed by Rossmann and Blow (Rossmann & Blow, 1962), and facilitated by Crowther (Crowther, 1971), rotation search

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Table I: Heavy-Atom Positions and R Values

heavy-atom compounds	R modulus	R weighted	sites	x	y	z
$KAuCl_4$	32	12	1	0.52	0.00	0.61
			2	0.50	0.04	0.04
K_2PtCl_4	41	28	1	0.52	0.00	0.11
			2	0.51	0.05	0.63
			3	0.89	0.02	0.67
			4	0.16	0.00	0.53
$HgCl_2$	48	27	1	0.40	0.00	0.71
			2	0.35	0.00	0.19
			3	0.55	0.10	0.05
$Pd(NH_3)_2Cl_2$	48	14	1	0.72	0.45	0.45
			2	0.24	0.45	0.33
			3	0.30	0.45	0.96
			4	0.78	0.45	0.96

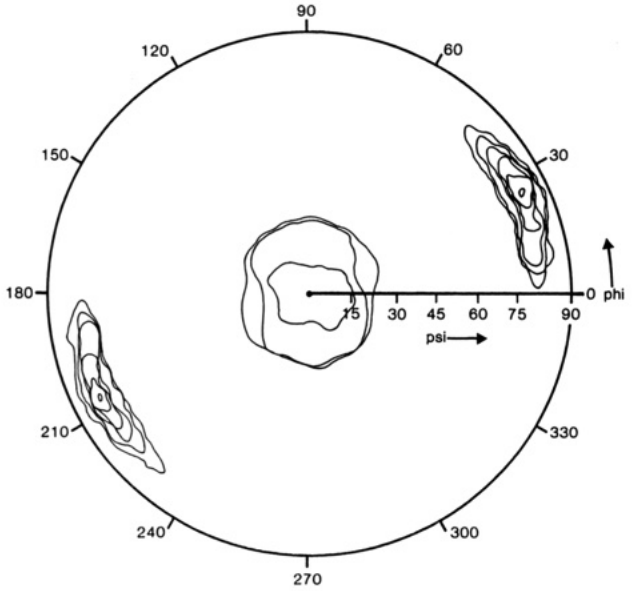

FIGURE 1: Stereo diagram of the rotation function performed on monellin native data. Rotation search was performed by varying two of the spherical angles (ψ, ϕ) by 5° intervals and the third (χ) by a 30° interval. The maximum values were obtained at $\chi = 180°$, $\psi = 90°$, and $\phi = 25°$. A finer interval of 1° was then used for ψ and ϕ to obtain this figure. The highest counter line is 21 and the lowest 6, with equal increments of 3 on a relative scale. χ is equal to 180°. The crystallographic a axis is horizontal, the b axis is perpendicular to the plane of the paper, and the c axis is at $\phi = 107.5°$. The final maximum is at $\chi = 180°$, $\psi = 84°$, and $\phi = 27°$.

calculations were carried out at 5° increments in two (ψ and ϕ) of the spherical coordinate parameters. After the approximate local rotation axis was found, a finer search was made by using 1° increments. The local twofold axis was found to be at $\psi = 88°$ and $\phi = 27°$ (see Figure 1), which is approximately perpendicular to the crystallographic b axis and forms an angle of 27° with the a axis.

Results and Discussion

The electron density map was quite clear, and it was easy to distinguish the molecular envelope in an asymmetric unit. Further examination of the map showed that the entire molecular envelope can be divided into two parts, each related to the other by a local twofold axis. The direction of this local twofold axis agreed with the result of rotation search performed on the native structure factors.

Figure 2 shows a photograph of a Styrofoam model of a monellin molecule as it appears in the asymmetric unit. The local twofold axis is perpendicular to the plane of the page, relating the portion of the asymmetric unit in white to the dark portion. Each portion represents two monellin molecules, and each molecule consists of two peptides of unequal length. As is apparent in this photograph, the two dimers are very similar. However, the examination of individual dimers gave no clues as to the possible presence of additional local twofold axis relating individual monomers.

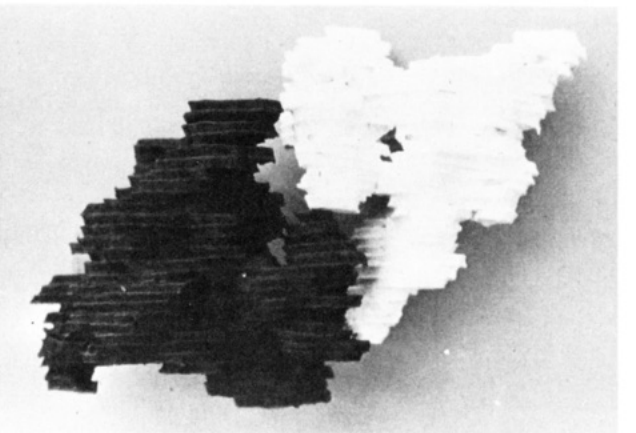

FIGURE 2: Model at 5.5 Å of four monellin molecules in an asymmetric unit. The crystallographic b axis is vertical. The local twofold axis is nearly perpendicular to the plane of the page, relating one dimer in the dark portion to the other dimer in the white portion.

The significance of dimer formation in the crystal is not obvious at the present time. Gel filtration chromatography performed on a calibrated Bio-Gel P-30 column as well as a sedimentation equilibrium study using a Beckman Model E analytical centrifuge showed (unpublished data) that monellin *at low concentration* exists in a monomeric state, having a molecular weight of approximately 10 250. However, there appears to be a slight affinity between monomers in *high* concentration: When we performed cross-linking experiments using suberimidate under high concentrations of monellin in solution, we found a small amount of cross-linking occurs between monellin monomers (unpublished data). Thus, the formation of dimer in the crystal presumably is not an intrinsic property of monellin in *dilute* solution but, rather, a property of concentrated monellin solution and due to crystal packing. Whether a sweet receptor recognizes monellin monomer or dimer is not known.

Examination of the monellin structure at 5.5-Å resolution does reveal many protruding features which are unlike most known globular protein structures. It is tempting to speculate that these protrusions may be the sweet receptor binding site. For high-resolution studies, we plan to average electron densities according to the local, noncrystallographic symmetries to improve the interpretability of the electron density map.

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Amplification and Organization of Dihydrofolate Reductase Genes in a Human Leukemic Cell Line, K-562, Resistant to Methotrexate†

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ABSTRACT: A subline of human leukemia cells (K-562), highly resistant to methotrexate, was developed by stepwise selection in the presence of increasing concentrations of this drug. The ED_{50} of these resistant cells was 1 mM compared to 10 nM for the parental line. Comparison of certain folate-requiring enzymes from crude extracts of the parent and resistant cells showed a 240-fold elevation of dihydrofolate reductase activity in the resistant cells with no significant increase in the levels of the other enzymes. Purified dihydrofolate reductase from the resistant cells had the same physical and kinetic properties as the enzyme from the sensitive cells. Southern blot analysis showed a marked increase in the number of dihydrofolate reductase genes in the resistant line. The genomic organization of the human dihydrofolate reductase gene was determined by hybridization with specific cDNA sequences from a human cDNA to DNA fragments from K-562 cells generated by restriction endonucleases. The human dihydrofolate reductase gene contained at least four intervening sequences and was approximately 30 kb in size. Northern blot studies demonstrated an increase of dihydrofolate reductase mRNA species; the predominant message was 3.8 kb. Karyotype analysis revealed three elongated marker chromosomes, derived from chromosomes 5, 6, and 19 which contained homogeneous staining regions, which were not present in the parent cell line.

Methotrexate is an important drug used in the clinic for the treatment of several human malignancies (Johns & Bertino, 1973). Development of drug resistance, however, occurs frequently and limits its further use. An understanding of the mechanism(s) of resistance to this drug in human cells might provide some insights into strategies to prevent resistance or to develop new therapies that selectively kill resistant cells (Bertino, 1979).

Amplification of the dihydrofolate reductase (DHFR)[1] gene is a known mechanism of resistance to methotrexate (MTX) in several mammalian cell lines (Alt et al., 1978; Bostock & Clark, 1980; Dolnick et al., 1979; Melera et al., 1980; Milbrandt et al., 1981). The presence of a homogeneous staining region (HSR) on a chromosome(s) has been associated with stable or slowly reversible drug resistance (Berenson et al., 1981; Biedler et al., 1980; Dolnick et al., 1979; Nunberg et al., 1978) whereas unstable amplification has been associated with the presence of the DHFR genes on double minute chromosomes (Kaufman et al., 1979).

To determine whether human cell lines could also develop resistance by virtue of DHFR gene amplification, K-562 cells, derived from a pleural effusion of a patient with chronic

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[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); FAH_2, dihydrofolic acid; FAH_4, tetrahydrofolic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; dUMP, deoxyuridine monophosphate; pCMB, *p*-(chloromercuri)benzoic acid; HSR, homogeneous staining region; kb, kilobase; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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