The author would like to express his appreciation to Professor Ephraim Katchalski for his hospitality, to Professor Michael Sela for samples from his collection and for valuable discussion, to Professor Franz Sondheimer for the use of a spectrophotometer, and to Dr. Israel Schechter and Mr. Shmuel Shaltiel for valuable advice.

Abbreviations used are: pTR, poly-L-tyrosyl ribonuclease; pApTR-L, lightly alanylated pTR, i.e., poly-DL-alanyl-poly-L-tyrosyl ribonuclease; pApTR-H, heavily alanylated pTR; A(T,G)L, poly-DL-alanyl-poly(L-tyrosyl, L-glutamyl)-poly-L-lysine; (T,G)A-L, poly-(L-tyrosyl, L-glutamyl)-poly-DL-alanyl-poly-L-lysine; R, ribonuclease; pAR, poly-DL-alanyl ribonuclease; and pTpAR, poly-L-tyrosyl-poly-DL-alanyl ribonuclease.

* Alfred P. Sloan Fellow, 1960–1964. Address for reprints: Department of Chemistry, State University of New York, Stony Brook, N. Y.

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AN ISOMORPHOUS HEAVY ATOM SUBSTITUTION AT THE ACTIVE SITE OF GAMMA-CHYMOTRYPSIN

BY P. B. SIGLER, H. C. W. SKINNER, C. L. COULTER, J. KALLOS,* H. BRAXTON, AND D. R. DAVIES

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, BETHESDA, MARYLAND

Communicated by Norman Davidson, April 15, 1964

Gamma-chymotrypsin (γCHT) is an active modification of the bovine protease chymotrypsin[1] which has a molecular weight of approximately 25,000.[2] It can be crystallized from half-saturated ammonium sulfate solutions in the space group $P4_22_12$ with one molecule in the asymmetric unit.[3] Since there is evidence indicating that the enzyme molecules in this crystal are in an enzymatically active configuration,[4] a detailed crystallographic structure determination would be of great interest.

In this study we have prepared a single site heavy atom derivative of γCHT by crystallizing γCHT inhibited with p-iodobenzenesulfonyl fluoride (pipsyl-F, Fig. 1). The position of the heavy atom, iodine, in the unit cell was determined by comparing the X-ray diffraction data from crystals of pipsyl-γCHT and crystals of γCHT

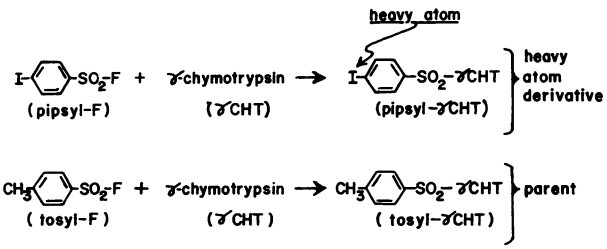

FIG. 1.—Schematic representation of the formation of two mutually isomorphous derivatives of γ-chymotrypsin.

inhibited with the analogous compound, p-methylbenzenesulfonyl fluoride (tosyl-F, Fig. 1). The use of tosyl-γCHT instead of the native enzyme as the parent protein clearly facilitated the location of the iodine atom by providing a higher degree of isomorphism. The isomorphous replacement was regarded as the stoichiometric substitution of an iodine atom for a methyl group.

The iodine atom is a substituent of a group which we have demonstrated to be linked irreversibly to the active site of chymotrypsin. The pipsyl-γCHT derivative therefore serves the dual purpose of providing a marker by which the active center of the enzyme may be localized crystallographically as well as providing a heavy atom derivative for the structure determination.

Materials and Methods.—Twice crystallized and lyophilized γCHT (Worthington Biochemical Corp.) was used without further purification. Para-iodobenzenesulfonyl chloride (pipsyl-Cl) from Mann Research Labs, Inc., was recrystallized once from n-hexane. I^{125}-labeled pipsyl-Cl (Volk Radiochemical Corp.) was recrystallized to constant specific activity with 3 gm of cold pipsyl-Cl.

Synthesis of p-methylbenzenesulfonyl fluoride (tosyl-F): Tosyl-F was prepared from tosyl-Cl by the method described by Fahrney and Gold[5] for the synthesis of similar sulfonyl fluoride esterase inhibitors. In this case, however, the product was crystallized from acetone by the addition of water (yield, 60%; m.p. 48–50°C; lit. m.p. 45°C; calculated: C, 48.3; F, 10.9; S, 18.4; found: C, 48.6; F, 11.0; S, 17.8).

Synthesis of p-iodobenzenesulfonyl fluoride (pipsyl-F): Four gm (13.3 meq) of pipsyl-Cl and 0.58 gm of NaF (13.8 meq) were stirred in 5 ml of dry dimethylformamide at 90°C for 30 min. The product was extracted with CH_2Cl_2 and recrystallized from cyclohexane (m.p. 83–84°, yield 32%). The product was further purified by sublimation (m.p. 86°C; calculated: C, 25.2; F, 6.6; S, 11.2; I, 44.3; found: C, 25.4; F, 6.6; S, 11.3; I, 44.0). Because of the lability of the ring-iodine bond, the procedure differs slightly from that outlined by Fahrney and Gold in that the conditions are less energetic and an excess of NaF is avoided. Pipsyl (I^{125})-F was prepared in an identical fashion using pipsyl(I^{125})-Cl.

Preparation of pipsyl-γCHT and tosyl-γCHT: The method employed is a modification of the procedure used by Fahrney and Gold[5] to inhibit chymotrypsin with phenylmethanesulfonyl fluoride. A 1% γCHT solution (4×10^{-4} *M*) in 0.05 phosphate buffer (pH 7.2) containing 10% 2-propanol was mixed with an equal volume of a 4.25×10^{-4} *M* sulfonyl fluoride solution in the same solvent and allowed to stand 2.5 hr at room temperature (99.0% inhibition). The protein solutions were lyophilized after extensive dialysis of the reaction mixture against 2×10^{-3} *M* cold acetic acid (pH 3.7). The inhibited enzyme was crystallized from a 1.25% protein solution half-saturated with $(NH_4)_2SO_4$. Pipsyl(I^{125})-γCHT was prepared in the same way using pipsyl (I^{125})-F. Derivatives prepared from the sulfonyl fluorides were found to be more suitable for the preparation of protein crystals used in these crystallographic studies than derivatives prepared from the corresponding sulfonyl-Cl using methods similar to those introduced by Kallos and Rizok[6] and Strumeyer *et al.*[7] for the tosylation of γCHT.

Enzyme concentrations were determined photometrically using $\epsilon_{2905\text{Å}} = 1.66$ mg ml^{-1} cm^{-1}. This extinction coefficient was used rather than the conventional $\epsilon_{2820\text{Å}}$ since the pipsyl contribution to the absorbance is significant for $\lambda < 2850$ Å.

The specific activity of I^{125} inhibitors was determined using standard solutions prepared by weighing out the solute (±0.2%). I^{125} activity was measured with a Packard automatic γ-ray spectrometer. Enzymatic activity was assayed by the method of Hummel.[8]

Crystallographic studies: X-ray diffraction photographs were taken with a Buerger precession camera using CuKα radiation. The [*h0l*] and [*hk*0] data were collected to a resolution of approximately 4 Å. Quartz calibrated photographs were used to determine the cell constants. The intensities of the reflections were determined with a Joyce-Loebl microdensitometer.

Results and Discussion.—Mole ratio of iodine:enzyme in crystals of pipsyl-γCHT: Crystals of pipsyl(I^{125})-γCHT were dissolved and exhaustively dialyzed. Enzymatic activity and protein bound I^{125} radioactivity were measured in the dialysate. If one radioactive pipsyl group is bound per molecule of enzyme, then the specific radioactivities of the inhibitor and the inhibited protein should be identical. The comparison of specific activity of pipsyl(I^{125})-F and pipsyl(I^{125})-γCHT presented below indicates that 0.98 atoms of iodine (as a pipsyl group) are irreversibly bound per molecule of enzyme (m.w. 25,000).[2] Furthermore, the fact that the enzyme has lost 99.3 per cent of its enzymatic activity as a result of this stoichiometric interaction indicates that essentially one iodine (as a pipsyl group) has been bound to *each* enzyme molecule.

Compound	m.w.	Counts × 10^{-7}/ min mmole	Iodine atoms/ enz. molecule	% Inhibition
Pipsyl(I^{125})-F	286	1.75		
Pipsyl(I^{125})-γCHT	25,000	1.72	0.98	99.3

The irreversible loss of enzymatic activity resulting from the stoichiometric interaction of pipsyl-F and γCHT suggests that pipsyl-F is covalently bonded to a residue at or near the active center. In addition we have demonstrated that pipsyl(I^{125})-F is prevented from reacting with γCHT previously inhibited by diisopropylfluorophosphate or tosyl-F (see below).

Gold and Fahrney[9] and Strumeyer *et al.*[7] have presented evidence that the inhibitors of chymotrypsin, phenylmethanesulfonyl fluoride, and p-toluenesulfonyl chloride, respectively, form sulfonyl esters with the serine previously implicated (Shaffer *et al.*,[10] Oosterbaan and van Adrichem[11]) as a part of the active center of chymotrypsin. In view of the close chemical similarity between these inhibitors and pipsyl-F it would seem reasonable to assume that pipsyl-F reacts with the "active serine" of γCHT. (See *Note added in proof.*)

Protein reactant	cpm/mmole × 10^7	Iodine atoms/enz. molecule
γCHT	1.83	0.96
DIP-γCHT	0.082	0.04
Tosyl-γCHT	0.162	0.08

Uptake of pipsyl (I^{125})-F by γCHT and two inhibited derivatives. Reaction time 80 min. Other conditions in text. Molecular weight of the enzyme = 25,000. Specific activity of pipsyl (I^{125})-F is 1.91 × 10^7 cpm per mmole.

Crystallographic studies: Unit cell dimensions of the native enzyme as well as the pipsyl and tosyl derivatives were determined and are given below.

Protein	*a*	*c*
γCHT	69.6	97.7
Pipsyl-γCHT	69.7	97.4
Tosyl-γCHT	69.7	97.4

Dimensions in Å units. Measurement accurate to 0.1%.

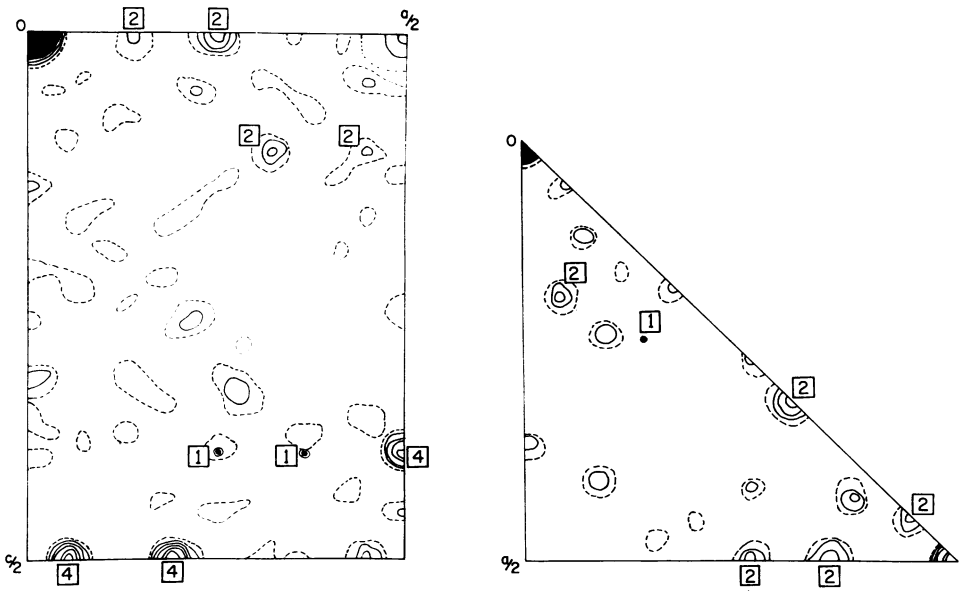

FIG. 2.—(*a*) Patterson projection, $P(u,w)$. Coefficients are $(|F_{\text{pipsyl-}\gamma\text{CHT}}|-|F_{\text{tosyl-}\gamma\text{CHT}}|)^2$. Predicted peak locations are indicated by the boxed numbers which represent the relative calculated weights. Contour intervals are equal and arbitrary; lowest contour dashed. Dots represent the expected positions of single weight peaks. (*b*) Patterson projection, $P(u,v)$. Coefficients and display as in (*a*).

The tosyl and pipsyl derivatives differ noticeably from the native enzyme mainly in the *c*-axis dimension. The two inhibited derivatives, however, have virtually identical cell constants thereby satisfying one requirement for strict isomorphism.

The position of the iodine atom was obtained by comparing the diffraction data of pipsyl-γCHT and tosyl-γCHT by means of the *a*-axis and *c*-axis difference Patterson projections shown in Figure 2*a* and *b*, respectively. A satisfactory interpretation of both maps can be made with the coordinates listed below. (Approximate values were derived from the Patterson projections and refined by the method of least-squares using the [*h*0*l*] data.)

$$x/a = 0.067 \qquad y/a = 0.376 \qquad z/c = 0.451$$

The relative peak heights observed are in reasonable agreement with the calculated weights based on a single site substitution. If the diffraction data of pipsyl-γCHT and the *native enzyme* are compared by means of similar Patterson projections, the resulting maps are not easily interpreted.

The Fourier map shown in Figure 3*a* represents the electron density distribution of the heavy atom (replacement) projected down the *a*-axis. It was computed using the differences between the observed structure amplitudes of the pipsyl and tosyl derivatives and the phases calculated from the coordinates of the iodine, as derived from the difference Patterson maps. Although the two heavy atoms in the asymmetric unit of this projection are clearly seen at (x,z) and $(y,\bar{z})$, such an electron density map cannot, by itself, be used to verify the coordinates because of the overriding effect of the assigned phases. However, this projection can be treated as if

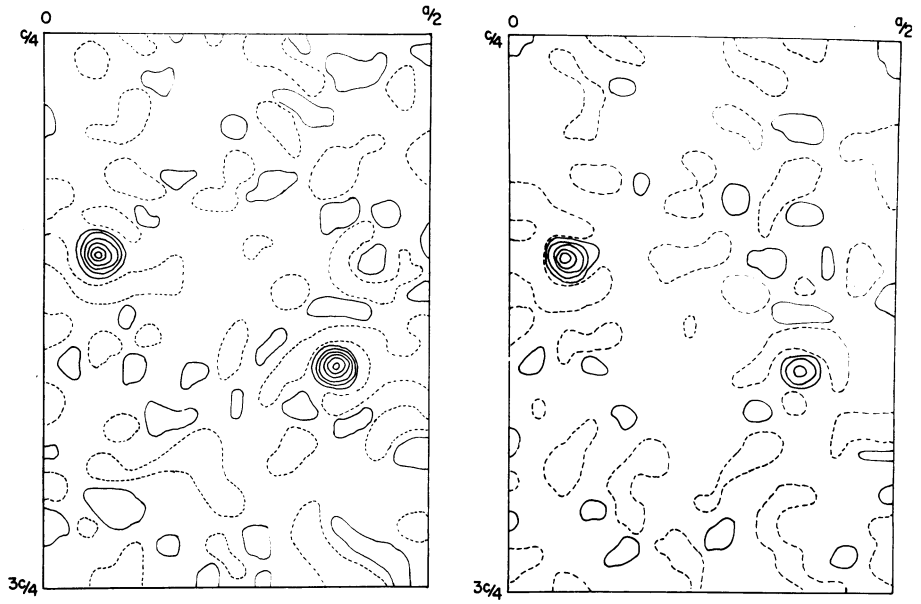

FIG. 3.—(*a*) Projected Fourier synthesis, $\rho(x,z)$. Phases based on coordinates of iodine atom listed in the text. Contour intervals are equal and arbitrary. Zero contour is dashed. Negative contours omitted. (*b*) Projected Fourier synthesis, $\rho(x,z)$. Phases based only on (x,z). Contour intervals are equal and arbitrary. Zero contour is dashed. Negative contours omitted.

it were orthorhombic for the purpose of calculating the phases, in which case either of the two positions can be introduced individually into the phase calculation. Thus, preliminary verification of the heavy atom coordinates was obtained by computing the *a*-axis Fourier projection phased with the (x,z) position alone (Fig. 3*b*). The $(y,\bar{z})$ peak remained (about $^1/_2$ the peak height of the (x,z) peak) even though it was not used in the calculation of the phases. Similarly, if the Fourier series was phased with only the $(y,\bar{z})$ position, the (x,z) peak remained (also about $^1/_2$ the peak height of the $(y,\bar{z})$ peak). In both cases the peak corresponding to the atom which was omitted from the calculation was at least double the highest background peak.

These results demonstrate that a crystalline heavy atom enzyme derivative of predictable stoichiometry can be produced in a chemically rational manner by taking advantage of the reactivity of the enzyme's active site. In addition, the localization of a heavy atom bearing a known chemical relationship to the active center may facilitate the structure determination of the active center. Some deformation of the protein structure no doubt accompanies the formation of an enzyme inhibitor complex (at least at the point of covalent interaction); therefore, we cannot state, at this stage, to what degree this method of crystallographically labeling the active center will facilitate the structure determination of the active center of the *native* enzyme.

Summary.—An irreversible inhibitor, p-iodobenzenesulfonyl fluoride, has been used to prepare a crystalline single site heavy atom derivative of γCHT which is isomorphous with crystalline p-toluenesulfonyl-γCHT. The coordinates of the heavy atom, iodine, have been derived from 4 Å difference Patterson projections.

Note added in proof: Since this manuscript was submitted, Kallos and Rizok[12] have obtained direct chemical evidence indicating that the pipsyl as well as the tosyl group is covalently bonded to a serine residue of γCHT.

We are indebted to Dr. Allen Gold for the gift of phenylmethanesulfonyl fluoride used to prepare PMS-γCHT in the early stages of this work.

* Research Laboratory, St. Mary's Hospital, Montreal, Quebec.

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*SELECTIVE INHIBITION OF SYNTHESIS OF RIBOSOMAL RNA IN ESCHERICHIA COLI BY LEVORPHANOL**

BY ERIC J. SIMON† AND DINA VAN PRAAG

DEPARTMENT OF MEDICINE, NEW YORK UNIVERSITY SCHOOL OF MEDICINE, AND THE SUMMER RESEARCH INSTITUTE OF THE WILL ROGERS HOSPITAL AND O'DONNELL MEMORIAL LABORATORIES, SARANAC LAKE, NEW YORK

Communicated by Colin M. MacLeod, February 28, 1964

We have previously reported that levorphanol, a close structural analogue of morphine, inhibits RNA synthesis in *E. coli* to the extent of 80–90 per cent.[1, 2] Since ribosomal RNA comprises 80–85 per cent of the total RNA of the cell, these results indicate that the synthesis of ribosomal RNA must be markedly inhibited by levorphanol. The present experiments were designed to determine the extent to which levorphanol inhibits the synthesis of messenger and transfer RNA. Evidence will be presented which demonstrates that levorphanol inhibits predominantly the synthesis of ribosomal RNA. This is in contrast to the findings with actinomycin D which has been shown to inhibit equally the synthesis of all types of cellular RNA.[3, 4]

Materials and Methods.—Bacterial strains and media: *E. coli* K-13, a prototrophic isolate of strain K-12, and Hfr Hayes, a methionine requiring K-12 "relaxed" mutant, were used in the present experiments. Cells were grown in minimal medium buffered by 0.05 *M* triethanolamine at pH 8.2, supplemented with 0.5% sodium succinate and 0.2% casamino acids as sources of carbon as described previously.[2] For the "step-down" experiment the enriched medium was prepared by adding 1% Difco Bacto-peptone to the minimal medium and readjusting the pH to 8.2.

Materials: O-nitrophenyl β-galactoside (ONPG) and isopropylthiogalactoside (IPTG) were purchased from Mann Laboratories, DNAase from Worthington Chemical Co. Levorphanol tartrate and levallorphan tartrate were made available through the generosity of Hoffmann-La Roche, Inc., Nutley, N.J. C^{14}-uracil was purchased from New England Nuclear Corp.