THE REACTION OF DIISOPROPYLFLUOROPHOSPHATE WITH CRYSTALS OF γ-CHYMOTRYPSIN

Paul B. Sigler and H. Catherine W. Skinner

Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

Received August 23, 1963

We wish to report that crystalline γ-chymotrypsin (γCT) is capable of reacting stoichiometrically in the solid state with diisopropylfluorophosphate (DFP) to give a product crystallographically indistinguishable from diisopropylphosphorylchymotrypsin (DIP-γCT) prepared in solution. When X-ray diffraction patterns of crystalline γCT and DIP-γCT are compared, they appear very similar; however, small changes in the unit cell dimensions (less than 0.4%) as well as small intensity differences are noted.[1]

Preparation of Crystals. Crystals of γCT and DIP-γCT suitable for X-ray diffraction studies were prepared as follows: a 1.25% solution of γCT (Worthington, salt-free, 2 X crystallized) was crystallized at room temperature from half saturated $(NH_4)_2SO_4$ solutions in 2-5 days at several pH's in the range 5.2 - 6.8, giving crystals 0.1-0.2 mm X 0.1-0.2 mm X 0.5-1 mm. DIP-γCT, prepared in solution by the method of Naughton et al. (1960), was crystallized under identical conditions. The mother liquor can be replaced by protein-free supernatant at the appropriate pH and $(NH_4)_2SO_4$ concentration without causing changes in the crystal morphology or diffraction pattern.

[1] Blow (1960) has crystallized the closely related protein, DIP-αCT, and found that the diffraction pattern of the inhibited enzyme is almost indistinguishable from that of the native enzyme.

This stability makes it possible to replace the mother liquor with supernatant containing other reagents, such as DFP and heavy metals, and then to recover and examine the crystals.

Reaction of Crystalline γCT with DFP. The inhibition of crystalline γCT was carried out in vials containing approximately 10 mg of crystalline γCT in protein-free 65% saturated $(NH_4)_2SO_4$. The crystals were exposed to a twentyfold molar excess of DFP (Sigma) by replacing the supernatant in each of the vials with 2 ml of a 4×10^{-3} M DFP solution in 65% saturated $(NH_4)_2SO_4$, buffered at pH 6.5 with 0.1 M sodium cacodylate, and containing 1% 2-propanol. Table I summarizes the details of the experiment. At the intervals indicated the supernatant was decanted and replaced with freshly prepared DFP solution. The reaction was stopped by removing the DFP-containing supernatant. The crystals were then placed in 65% saturated $(NH_4)_2SO_4$, pH 5.2, for 24 hours to allow unreacted DFP to diffuse out of the crystals. After removal of several crystals for X-ray diffraction studies, the remaining crystals were dissolved in 7 ml of 2×10^{-3} M acetic acid (pH 3.7) at 0° C. The low temperature and pH served to prevent any residual DFP from reacting with the redissolved enzyme. 5-100λ aliquots of this solution were assayed for enzymatic activity, using a buffered solution of benzoyl-L-tyrosine ethyl ester (BTEE) as substrate (Hummel, 1959). Protein concentrations were determined photometrically (Schwert and Takenaka, 1955). Several vials of crystals exposed to the reaction conditions described above, but with DFP omitted, served as controls for both the X-ray diffraction studies and enzyme activity assays.

As shown in Table I, the γCT crystals exposed to DFP for 144 hours were 97-99% inhibited. Furthermore, the stoichiometry of the reaction was studied using DFP labeled with H^3 in the C_1 of the isopropyl group (New England Nuclear Corp.), and the results indicate that between 0.85 and 0.92 moles of DFP are bound per mole of crystalline γCT inhibited.

TABLE I

Inhibition of Crystals of γCT by DFP

Vial	Time (hours) 0	3.5	22	26	44	50	144	% Inhibition
1	+	+	+	+	R			86
2	+	+	+	+	+	+	R	97
3	+	+	+	+	+	+	R	99
4_c	$+_c$	$+_c$	$+_c$	$+_c$	R			0
5_c	$+_c$	$+_c$	$+_c$	$+_c$	$+_c$			0

\+ Indicates removal of supernatant and addition of freshly prepared DFP-containing solution.

$+_c$ Indicates a similar change of supernatant solutions as "+" but without DFP.

R Indicates crystals removed for X-ray diffraction and enzyme activity studies.

The relatively slow rate of reaction between γCT crystals and DFP (86% inhibition in 44 hours) may be the result of many factors. Two possibilities, the high $(NH_4)_2SO_4$ concentration and the diffusion rate of DFP into the crystals, were qualitatively evaluated.

The second order rate constant for the inhibition of γCT solutions by DFP was found to be increased by a factor of 5 when the $(NH_4)_2SO_4$ concentration was raised from 25% to 65% of saturation. Similarly, over the same range of $(NH_4)_2SO_4$ concentration the enzymatic hydrolysis of the specific substrate, BTEE, was increased 19%.[2] Presumably a high concentration of $(NH_4)_2SO_4$ in the crystals would not, therefore, be expected to affect the enzyme's activity adversely.

[2] γCT, in the low concentrations used in these experiments, remains in solution over this range of $(NH_4)_2SO_4$ concentrations.

The rate of diffusion of DFP into the crystals was examined by reacting large (0.8 x 0.8 x 2 mm) crystals and small (0.06 x 0.06 x 0.15 mm) crystals with DFP under identical conditions. After 20 hours the large crystals were 8% inhibited whereas the small crystals were 25% inhibited, indicating that diffusion is probably the rate limiting step in the reaction of crystalline γCT with DFP.

Since the reactivity of γCT with DFP can be correlated with enzyme activity (Balls, A. K. and Jansen, E. F., 1952; Hartley, B. S., 1956), the reaction of DFP with crystals of γCT suggests that the crystalline enzyme is in an enzymatically active conformation.

X-Ray Diffraction: Methods and Results. X-ray diffraction photographs of γCT and DIP-γCT crystals were taken with a Buerger single crystal precession camera using CuK_{α} radiation. Both proteins gave equally well resolved photographs at spacings of less than 2 Å, indicating very little, if any, breakdown of the short range order in the DIP-γCT structure. Precession photographs used for comparisons exhibited sharp and well defined diffraction maxima to a resolution of 3.5 Å. Comparative cell dimension measurements were made on quartz calibrated films with the aid of a Charles Supper precession film measuring device and checked on microdensitometer tracings (Joyce Loebl microdensitometer).

The DIP-γCT crystals show an increase of 0.4% in the a axial direction and a decrease of 0.2% in the c axial direction relative to γCT.

X-ray diffraction photographs of the reaction product of DFP and crystals of γCT (97% inhibited) described above are indistinguishable from those of DIP-γCT previously prepared in solution.

The DIP-γCT photographs exhibited small but reproducible intensity changes when compared with the patterns of the native enzyme. Intensity differences may arise from the isomorphous insertion of a DIP group into the structure as well as from small conformational

changes. It is difficult to state to what extent the changes in intensity noted here reflect conformational changes in the enzyme. The unequivocal interpretation of such changes must therefore await the detailed structure analysis of the enzyme. It is evident, however, that any changes in the conformation of γCT induced by the reaction of the enzyme with DFP are sufficiently small so as to leave the crystal lattice essentially undistorted.

Evidence supporting the existence of enzymatic activity in crystalline ribonuclease-S has recently been reported. Doscher and Richards (1963a,b), by analysis of substrate turnover rates, have inferred that crystalline ribonuclease-S in 90% saturated $(NH_4)_2SO_4$ is enzymatically active. As in the studies reported here, diffusion is the rate limiting step, and X-ray diffraction intensity differences are noted with essentially no disruption of the crystal lattice.

ACKNOWLEDGMENTS

We wish to thank Dr. Jean Kallos for his help and advice with some of the early experiments and Drs. G. Tomkins and D. Davies for helpful advice.

REFERENCES

Balls, A. K., and Jansen, E. F., Adv. in Enzymol. 13, 321 (1952).
Blow, D. M., Personal communication (1960).
Doscher, M., and Richards, F. M., Fed. Proc. Abst. 22, 1542 (1963a).
Doscher, M., and Richards, F. M., J. Biol. Chem. 238, 2399 (1963b).
Hartley, B. S., Biochem. J. 64, 27p (1956).
Hummel, B. C., Can. J. Biochem. and Physiol. 37, 1393 (1959).
Naughton, M. A., Hartley, B. S., Sanger, F., and Shaw, D. L., Biochem. J. 77, 149 (1960).
Schwert, G. W., and Takenaka, Y., Biochim. et Biophys. Acta 16, 570 (1955).