Thiamine Pyrophosphate Hydrochloride: Stereochemical Aspects from an X-Ray Diffraction Study

Abstract. *The crystal structure of thiamine pyrophosphate has been determined by a three-dimensional x-ray analysis. The conformation of the molecule in the crystalline state is determined by the formal charge distribution within the molecule which exists as a zwitterion with the negative pyrophosphate chain folded back over the positive, ring portion of the molecule. The oxygen atoms in the pyrophosphate group are in the staggered conformation when viewed along the phosphorus-phosphorus axis. Even though the pyrophosphate is present in this compound as the monoionized monoester, the configuration is the same as that present in the inorganic pyrophosphate ion. From a comparison of three different crystal structures containing the thiamine moiety and from studies with atomic models, it seems plausible that the basic molecular conformation observed in this crystal is maintained in the catalytically active molecule. Knowledge of the detailed crystal structure provides new insight into the biochemical mechanism of reactions catalyzed by thiamine pyrophosphate.*

The detailed three-dimensional crystal structure of thiamine pyrophosphate (cocarboxylase) is of general biochemical importance because this compound serves as the coenzyme in numerous enzyme systems concerned with the transfer of an "active aldehyde" moiety and because it provides stereochemical information that is useful for understanding the biochemical mechanisms of these systems. Moreover, the structural parameters of the pyrophosphate group are significant since it is the energy source for many biochemical reactions, even though in thiamine pyrophosphate it does not function as an energy source in any known biological system.

Single crystals of the coenzyme were grown from an ethanolic 0.1*N* HCl solution near 0°C. They are monoclinic and have the space group symmetry $P2_1/c$. The unit cell dimensions, as obtained from oscillation and Weissenberg photographs, are $a = 6.92 \pm 0.02$, $b = 11.28 \pm 0.05$, $c = 24.82 \pm 0.10$ Å and $\beta = 93°48' \pm 15'$. There are four molecules of thiamine pyrophosphate hydrochloride per unit cell. The density as measured by flotation in a mixture of chloroform and methylene bromide is 1.68 g cm^{-1}. The chemical assay (*1*) requires two molecules of water per unit cell which corresponds to a calculated density of 1.61 g cm^{-1} based upon the x-ray data.

The crystal structure was determined from three-dimensional x-ray single-crystal intensity measurements. The diffracted intensities were recorded by the equi-inclination technique and estimated by visual comparison with a calibrated film strip.

Thiamine pyrophosphate is present in the crystal as a zwitterion containing one negative and two positive charges. Protonation of the N-1 nitrogen in the pyrimidine ring gives rise to one of the positive charges. The other is associated with the thiazolium ring. The negative charge is distributed over the oxygen atoms of the pyrophosphate group, the largest partial negative charges being on the oxygen atoms of the three shortest P—O bonds. The net charge on the zwitterion is balanced by that of the chloride ion. The structure of the molecule and its mode of packing in the unit cell are shown in Fig. 1. The conformation of the molecule is quite likely determined to a large extent by the formal charge distribution within the molecule itself. By folding the negative pyro-

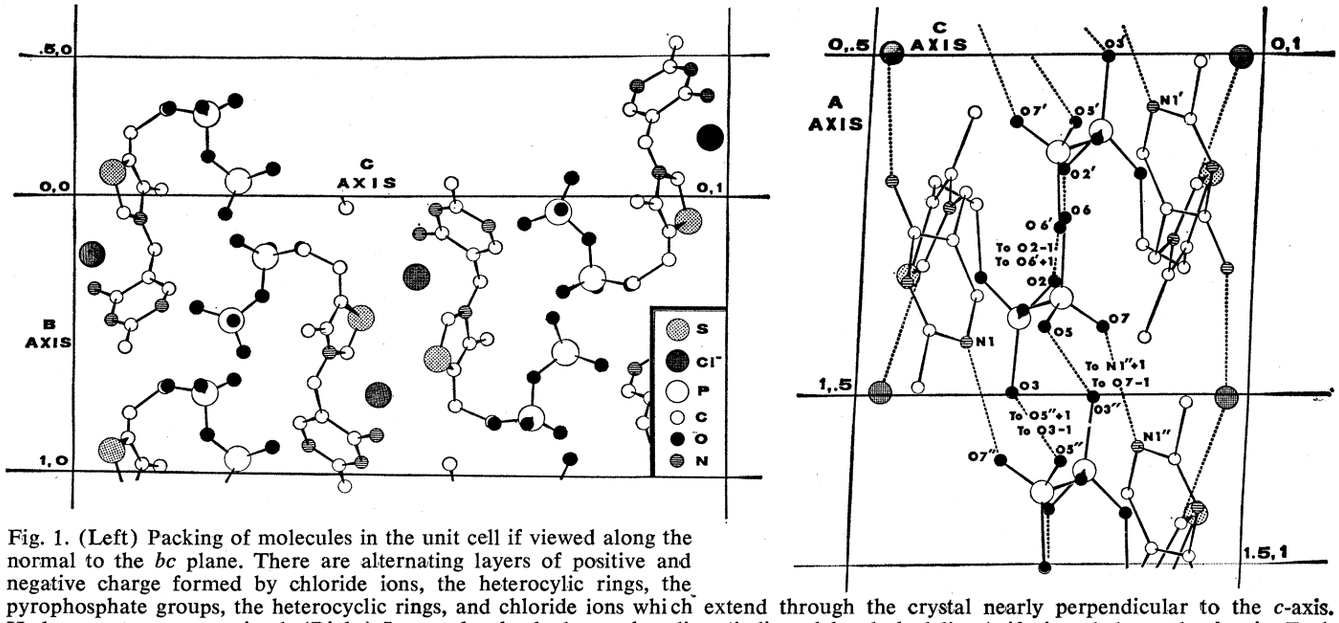


Fig. 1. (Left) Packing of molecules in the unit cell if viewed along the normal to the *bc* plane. There are alternating layers of positive and negative charge formed by chloride ions, the heterocylic rings, the pyrophosphate groups, the heterocyclic rings, and chloride ions which extend through the crystal nearly perpendicular to the *c*-axis. Hydrogen atoms are omitted. (Right) Intermolecular hydrogen bonding (indicated by dashed lines) if viewed down the *b*-axis. Each molecule is hydrogen-bonded through the pyrophosphate group to four different molecules all of which are equivalent by the identity translation along the *a*- or *b*-axes, or both. Bonds to atoms in molecules which are translated by one unit along the *b*-axis are designated by ± 1 (positive *y* extends behind the plane of the paper). Hydrogen atoms omitted. Atoms O-5 and O-6 of the terminal phosphate are bonded covalently to hydrogen atoms. The atom designations are the same as those in Fig. 1 (left), but the scale of the two drawings is different.

phosphate chain over the positive ring portion of the molecule, the separation of unlike charges is minimized.

The molecules are held together in the crystal by ionic attractions and hydrogen bonding to form a relatively open structure. The intermolecular hydrogen bonding occurs between the pyrophosphate groups and between the pyrophosphate and the protonated nitrogen of the pyrimidine ring. In addition, the chloride ion is hydrogen bonded to both the amino nitrogen and the thiazolium-ring carbon bearing the hydrogen atom. The intermolecular bonding involving the pyrophosphate groups and the ring nitrogen produces an irregularly-shaped cavity which extends through the crystal in the direction of the *a*-axis. Evidence at present indicates that there are at least two molecules of water in the unit cell. However, we could not locate any water molecules in the difference Fourier synthesis even though all of the hydrogen atoms in the thiamine pyrophosphate molecule were located by this technique. We believe that the water molecules are disordered in these cavities.

Further support for this idea can be obtained with the aid of space-filling models (*2*). By constructing a portion of two neighboring unit cells consisting of four intermolecularly bonded molecules, the existence of the cavity becomes readily apparent. With this model a water molecule can be accommodated at several different locations inside the cavity within a single unit cell. The two halves of the unit cell along the *z* direction which are related by a center of symmetry form a pocket which contains the chloride ion. Although the individual coenzyme molecules contain no asymmetric centers, the molecules in the two halves of the cell which are related by a center of symmetry are nonsuperposable mirror images by virtue of their molecular conformations. Conversion from one enantiomorphic form to another merely requires rotations about single bonds. It is interesting that the intermolecular hydrogen bonding occurs only between molecules of the same configuration: right-handed with right-handed, and left-handed with left-handed. These two hydrogen-bonding networks exist side by side and are held together by ionic attractions between alternate layers of positive and negative charge which are aligned nearly perpendicular to the direction of the *c*-axis (Fig. 1*a*).

Pyrophosphate is present in this crystal structure as the dihydrogen pyrophosphate monoester. Interestingly, the two nonionized protons are bonded to the terminal phosphate. The bond lengths and valency angles within the pyrophosphate group are shown in Fig. 2. The three shortest P—O bonds, averaging 1.50 Å, are equal to within the experimental accuracy of this determination which implies that they bear equal partial negative charges. Inasmuch as no other detailed structure analysis of an organic pyrophosphate molecule has been reported, it may be relevant to point out that the distances and angles found in this pyrophosphate monoester are similar to those of chemically equivalent bonds and angles in inorganic pyrophosphates (*3*) and organic monophosphate esters (*4*, *5*). In addition, the staggered conformation which is observed in the inorganic pyrophosphate structures is also found here. This can be clearly seen from the hexagonal distribution of the oxygen atoms when the pyrophosphate group is viewed along the line joining the centers of the phosphorus atoms. In this view, the pyrophosphate bridge oxygen projects midway between two of the hexagonally distributed oxygens, and the three multiply bonded oxygens are on the side opposite to the bridge oxygen. Since this conformation does exist in both inorganic and organic pyrophosphates, it very likely represents the most stable conformation even though this can be influenced in the crystalline state by intermolecular interactions. On these grounds we would therefore predict that the structure of the pyrophosphate group in other monoionized pyrophosphate monoesters is similar to that observed here.

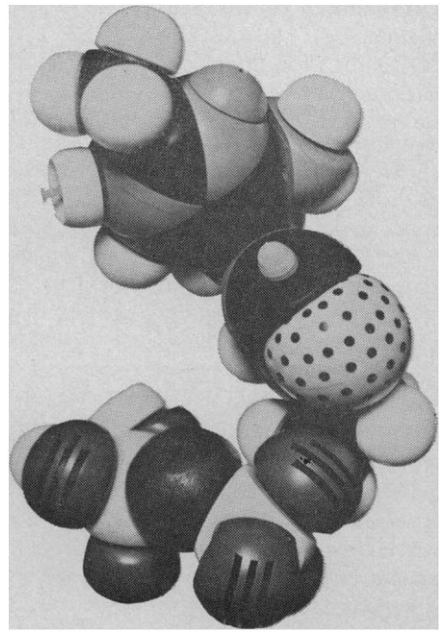


Fig. 3. Model of a single molecule with the three multiply bonded pyrophosphate oxygen atoms resting on a surface. The reactive site on the molecule (the carbon atom just above and to the left of the sulfur which is stippled) points away from the supporting surface. The hydrogen atom has been removed to show more clearly the bond direction. The amino substitutent on the pyrimidine ring is situated directly above the active carbon atom.

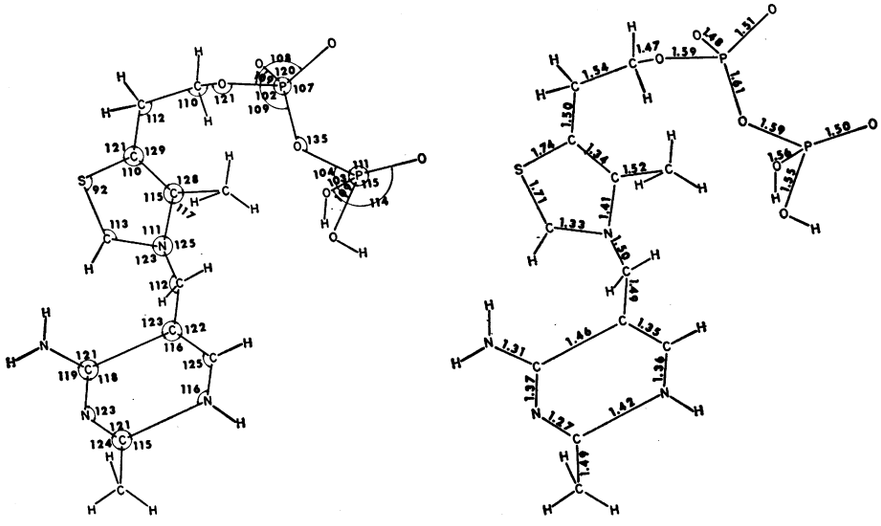
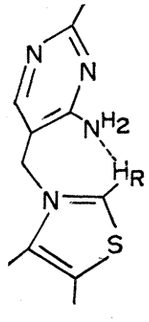


Fig. 2. Valency angles (degrees) and bond lengths (Å) obtained from final refinement.

A comparison of the structures of thiamine (*6*), thiamine monophosphate (*4*), and thiamine pyrophosphate indicates that there is some flexibility in the preferred orientation between the two rings since the dihedral angles (the angle between the normals to the planes of the two rings) in these structures are 76°, 90°, and 84°, respectively. However, since the conformation of the rings in the three structures is basically similar, this is the likely conformation in the catalytically active molecule. According to Breslow's mechanism (*7*) the initial step in the reaction is the loss of a proton from the thiazolium ring. Part of the support for this mechanism rests on the observed acidic nature of this thiazolium ring hydrogen. In all three crystal structures containing the thiamine moiety, the thiazolium carbon which contains the active hydrogen participates as a hydrogen bond donor, an unusual role for carbon, which demonstrates its acidic character. In the subsequent steps of the proposed mechanism the substrate reacts with the resulting carbanion to form several different intermediates before the final product is released. By the use of space-filling models it is possible to build these intermediates with the rings in the above-mentioned conformation. From these models it can be shown that formation of the intramolecular hydrogen bond is possible between the amino group and the substrate group as is illustrated diagrammatically below.



In order to achieve the most favorable spatial relationships for this intramolecular hydrogen bond formation, it is necessary to rotate the rings slightly (~10° to 15°) about both bonds to the methylene carbon and also to rotate the amino group slightly. The amount of rotation required depends upon the nature of the intermediate attached to the thiazolium ring and the specific hydrogen involved in the hydrogen bond formation. The structural possibility of forming these intramolecular hydrogen bonds may be significant in light of the suggestion by Breslow and McNelis (*8*) that several of the steps in the reaction sequence may be assisted by internal proton removal. The main conformational difference in the three structures is found in the dimethylene side chain to which the pyrophosphate group is attached. In thiamine pyrophosphate the dimethylene group extends to the opposite side of the thiazolium ring from that found in the other two structures.

Another structural feature of the thiamine pyrophosphate molecule worth noting is that the three nonprotonated pyrophosphate oxygens are directed away from the same side of the molecule. If a model of the molecule is supported on a flat surface by these three atoms, the remainder of the molecule is directed away from that surface (Fig. 3). In particular, the reactive site on the thiazolium ring points away from this surface. Since the pyrophosphate group apparently serves to bind the molecule to the enzyme, the structural arrangement observed here would allow the coenzyme to be bound to the protein surface and yet permit the reactive site to be freely accessible to the substrate. Also, small relative conformational shifts between the two rings can be readily accomplished as may be required for the various different intermediates in the reaction.

Because the crystals used in this analysis were very small, only about two-thirds of the reflections within the copper sphere could be measured. With this data it was possible to refine the structure anisotropically to a final agreement index (R factor) of 12 percent. At this stage of the refinement the estimated standard deviation in the P—O bond lengths is 0.010 Å whereas for the C—C and C—N bonds the average value is approximately 0.015 Å. Since it is desirable to know the structural parameters more accurately, the intensity data are being remeasured with a diffractometer from a somewhat larger crystal. The complete structure analysis utilizing the improved data will be published later.

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References and Notes

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9. We thank F. Rial for his assistance in preparing the illustrations. Supported by NIH grant HE-09068.

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Early Eocene Bat from Wyoming

Abstract. *A fossil skeleton of an early Eocene bat, the oldest known flying mammal, was found in southwest Wyoming. The bat is assigned to the new species* Icaronycteris index *of the suborder Microchiroptera. It was apparently of a young male whose body was buried in varved marls of the Green River Formation, on the bottom of Fossil Lake, about 50 million years ago. The bones, some as slender as a human hair, show a few "primitive" characteristics such as a clawed index finger and a complete phalangeal formula, but the bat was fully developed —an anatomically precocious contemporary of the dog-sized polydactylous horse.*

Remarkably complete and beautifully preserved, the skeleton of an early Eocene bat (see cover) from southwest Wyoming represents the oldest-known genus and species, here named *Icaronycteris index,* within the order Chiroptera. Found about 33 years ago (*1*) in the fossil-fish marlstones of the Green River Formation, the specimen is one of the most extraordinary vertebrate fossils in both the superb completeness of the bony skeleton and the rare preservation of remnants of structures such as wing membranes, abdominothoracic diaphragm, and cartilages of the ribs and throat—even of fragmentary residues of ingested food.

I now report for the first time some of its unique characteristics. Living bats are so numerous, in contrast with