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An X-ray Diffraction Analysis of Inhibitor Binding to Glycogen Phosphorylase a.

by

© Daniel Shun-Chung Yang

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

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Department of Biochemistry

EDMONTON, ALBERTA

Spring 1980

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled An X-ray Diffraction Analysis of Inhibitor Binding to Glycogen Phosphorylase a. submitted by Daniel Shun-Chung Yang in partial fulfilment of the requirements for the degree of Master of Science in Bicchemistry.

Robert Fletterick

Supervisor

Linda N. Johnson

Linda N. Pidusko

Date 26th Nov 1979

Abstract

In this thesis, the crystallographic difference Fourier method is employed to investigate the interaction between the enzyme Glycogen Phosphorylase a and some of its inhibitors. The focus is on the "Nucleoside site". The interesting finding of an unusual stacking interaction between the enzyme and its inhibitors sheds light on the specificity of this site. The bioenergetics of the interaction are discussed as well as the mechanism of inhibition.

The fear of the Lord is the beginning of wisdom, and knowledge of the Holy one is understanding.

Proverbs 9:10

Making many books there is no end,
much study wearies the body.

Ecclesiastes 12:12

Acknowledgements

I am grateful to Dr. R.J. Fletterick for his supervision and encouragement throughout the course of this investigation. I wish to thank Drs. N.B. Madsen and S. Sprang for their helpful discussion. I wish also to thank Miss H.F. Wong for her help and discussion during the time of writing of this thesis.

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I. Introduction.

Glycogen phosphorylase plays a major role in various regulatory processes in glycogen metabolism. It degrades storage glycogen using inorganic phosphate to form glucose-1-phosphate for producing ATP through the glycolytic pathway. The enzyme responds to energy control via AMP and ATP, feed-back inhibition by glucose and glucose-6--phosphate, and a number of hormonal and nervous controls. In addition to its substrates glycogen, G-1-P and inorganic phosphate, it is also known to bind nucleosides and nucleotide derivatives, regulatory enzymes, divalent metals and glucose.

A substantial body of research has been done, especially concerning the structural and functional aspects of the enzyme. The enzyme is known to exist in two chemically and functionally distinct forms, Phosphorylase *a* (with phosphate on serine-14) and Phosphorylase *b* (without phosphate), the interconversion of the two forms is regulated by a special kinase and phosphatase. Phosphorylase *a* in the absence of ligands is partially in the active (R) form whereas phosphorylase *b* exists mainly in the inactive (T) form. The R-T equilibrium is regulated by various unknown control mechanisms, using either metabolites or macromolecules or both.

Both *a* and *b* forms of the enzyme from rabbit muscle have been investigated by crystallographic studies and the structures have been resolved at 2.5 angstroms and 3.0

angstroms respectively (Sprang and Fletterick 1979; Weber et al 1978). Distinct binding sites have been found for several of the inhibitors, activators and substrates. The functions of each of these sites and their effect on the R-T conversion are listed as follows:

Site/Ligands	State	Functions
AMP/ATP	R/T	Metabolic response to ATP/AMP level
Glucose/G-1-P	T/R	Inactivation/ activation of phosphorylase a
Nucleoside	T	Inactivation of phosphorylase a
Glycogen	R	Partial activation, dissociation of tetramer to dimer
Ser-14/(p)	T/(R)	Hormonal response

To understand the molecular mechanism of R-T conversion for any of the above entries(sites) is very difficult. One way to increase our understanding is to look into each of these sites crystallographically to investigate the interaction between effectors and the binding sites.

The purpose of this thesis is to study the nucleoside site by examining a number of fused ring compounds, with respect to its detailed interactions with the enzyme Phosphorylase a.

The method employed is known as difference Fourier synthesis. It was first used in protein crystallography by Stryer in 1964 to examine the binding of azide ion to

Myoglobin. Since then, it has been extensively used by protein crystallographers to investigate protein-ligand interactions, protein conformational changes, location of heavy atom binding sites etc.

The difference Fourier, Delta D, is defined by

$$\Delta D = \frac{1}{V} \sum m |\Delta F| \exp i\alpha \cdot \exp -2\pi i hx$$

where V = volume of unit cell

m = figure of merit (a measure of the correctness of the phases, =1 represents correct phases).

$\Delta F = F_{Pi} - F_P$

F_{Pi} = structure amplitude of the Protein complex

F_P = structure amplitude of the Parent

α = phases of the Parent structure amplitude

The power of the method lies in the fact that a large number of similar structures can be investigated by using only the parent's phases plus the individual structure amplitudes for the particular binding experiment. The resulting density map from this calculation represents the difference between the protein complex and the parent. In the case of ligand binding the peaks represent the ligand and the structural changes induced by the ligand with peak heights equal to half of their true values. A detailed decription of the method can be found in the book by Blundell and Johnson (1976).

Due to the extensive application of this method, it is important to look into its reliability and accuracy. This has been dealt with in great detail by Henderson and Moffat (1971). In their study they focused on errors in the difference Fourier maps from three sources:-

1) use of the difference Fourier coefficients Delta f instead of the true structure amplitude;
2) experimental errors in the Delta F values;
3) experimental errors in the phases of the parent structure amplitude.

The remarkable conclusion they have drawn is that the difference Fourier map would have a much lower error level than the corresponding Fourier map of the derivative structure (which would require a new complete MIR phase calculation).

A. Materials and Methods.

Crystallization

Single crystals of Phosphorylase *a* suitable for diffraction studies were kindly supplied by Dr. Madsen and S.Shechosky. They were prepared as the procedure outlined below.

Rabbit muscle Phosphorylase *b* was prepared by the method of Fischer and Krebs (1962) with minor modifications including the use of 5 mM-dithiothreitol in place of mecaptoethanol. Phosphorylase *a* was prepared from Phosphorylase *b* with kinase by the methods of Krebs *et al* (1964). After three crystallizations in a micro-needle form from 10 mM Sodium glycerophosphate, 5 mM Dithiothreitol, 1 mM EDTA (pH 6.7), the Phosphorylase *a* was transferred into 10 mM BES (N,N-bis(2-hydroxymethyl)-2 aminomethane sulphonic acid), 0.1 mM EDTA (pH 6.7), by passage through a Sephadex G25 column at 39 degrees. Portions of concentrated stock solutions of magnesium acetate, dithiothreitol and glucose were added to the protein solution to give final concentrations of 10, 3, and 50 mM, respectively.

The protein, at concentrations of 1.5 to 3 %, was placed in small glass tubes 5 cm long, 2.4 mm internal diameter, and sealed at both ends with Parafilm. Although Phosphorylase *a* would form tetragonal crystals spontaneously, a small portion of a highly diluted stock suspension of finely ground seed crystals were usually injected to control the number of nucleation centers.

Crystallization was allowed to commence at 22 degrees, with storage at 16 degrees after a few days. This crystallization procedure has been reported earlier.(Fletterick et al 1976).

The enzyme crystallizes in tetragonal space group P43212 having lattice parameters a=b=128.4 angstroms and c=116.6 angstroms with one monomer of 97,400 daltons in the asymmetric unit.

Materials

Caffeine, FMN Sodium salt and Inosine were obtained from Sigma Chemical Co., ATP Sodium salt was obtained from Terochem Co., ATP had been monitored by a Paper Chromatographic method to ensure no contamination of AMP. Ascending chromatography has been used as described by the Biochemicals reference guide (PL Biochemicals Inc. 1977). Buffer system No.1 (Isobutyric acid/conc.NH2OH/ Water in ratio of 66/1/33) was used.

Crystal soaking

The ligand-binding studies that are referred to in this report were carried out by soaking the protein crystals in a solution of standard buffer with added ligands. The standard buffer is 10 mM N,N-bis(2-hydroxymethyl)2-aminomethane sulphonic acid / 0.1 mM EDTA / 10 mM magnesium acetate, pH 6.7. The soaking time and ligand concentration are listed in Table 1.

Data Measurement

All data measurements were done on a modified Syntex Analytical instruments, P21 automatic diffractometer, as

TABLE 1
Crystals soaking and other conditions (see text).

	Caffeine	Inosine	FMN	ATP
No. of Crystals used	51	9	15	14
Total no. of observations	44009	11165	16809	16398
Total no. of reflections	21599	10351	11571	12615
Total overlaps	37590	1509	8234	6141
Merging R factor	0.025	0.035	0.049	0.022
Delta F/F	14%	5%	7%	6%
Radiation decay rate (percent per hour)	3.3%	1.3%	2.1%	2.9%
Lattice constants	128.0	128.2	128.2	128.2
(in Angstroms)	116.4	116.4	116.5	117.0
Concentration of ligand	2 mM	15mM	0.5mM	20mM

reported earlier (Fletterick et al 1976). The counter and X-ray source were positioned 55.0 cm and 28.5 cm, respectively from the crystal. The data were collected using Ni filtered Cu radiation with the X-ray tube powered at 2000W. The diffractometer was operated in the Omega-scan mode with 7 steps of 0.012 degrees scanned at the peak center. From these 7 measurements, the highest neighboring 4 were selected and summed.

The background correction was applied by analyzing the background variation as a function of PHI and TWO THETA. CHI dependence was not taken into account since the beam cross-section is circular. Two background data files were collected from each crystal, the first consists of a measurement of background intensity as a function of PHI at constant TWO THETA (12.0 degrees) and the other one consists of a measurement of background intensity as a function of TWO THETA. The measurements were made in between the lattice points by doubling the lattice parameters and measuring a

selected series of odd values of h,k,l's. Approximately 0.5 degree increments of TWO THETA and 10 degree increments of PHI at TWO THETA = 12.0 degrees were made, andthe Omega-scan technique was used as for the data collection.

Data Processing

Radiation damage was monitored by repeatedly measuring 10 strong reflections uniformly distributed between TWO THETA of 3 tc 29 degrees, and analysed as a function of time and TWO THETA using the decay model of Fletterick et al(1976), Variation as function of h,k,l was not been taken into acccunt.

Standard Lorentz and polarization corrections were applied as well as absorption correction (North and Phillips 1968) which was analysed as a function of PHI only.

Approximately half of the observable HKLs to 2.5 angstrom resolution were measured for the difference Fourier studies. These reflections were taken from lists of reflections that had been sorted according to the product of figure of merit, magnitude of parent structure amplitude and resolution (Sin THETA) squared. This procedure should give the best measured and best phased data with a constant distributicn with respect to resolution. Three lists were prepared according to this scheme representing reflections in the spherical shells of 4.5 - 20.0 angstroms, 3 - 4.5 angstrcms and 2.5 - 3 angstroms respectively. The top halves of these three lists (strongest F's) were used, and from them HKL data lists were prepared for each crystals.

About 15 crystals were used to complete one data set because a single crystal diffracts for about 14-16 hours before it decays severely. Thirty reflections were included in each data list for scaling together the data sets from each crystal. These reflections were evenly distributed throughout the reciprocal lattice. In many cases, a large degree of overlap of HKL's were allowed in two consecutive data lists. The data lists were merged and scaled using G.N.Reeke's scaling program. The merging R factor for crystals (Rc) is defined as

$$Rc = \frac{\sum_{hkl} \sum_{i} \left| I_{hkl}^{i} - \bar{I}_{hkl} \right|}{\sum_{hkl} \bar{I}_{hkl}}$$

Where I_{hkl} is the intensity after a weighted scaling of the ith measurement of the intensity of reflection hkl and $\bar{I}(hkl)$ is the mean value of the N estimates of this intensity from different crystals. The summation is taken over all equivalent reflections measured for each crystals. Those data lists which showed a poor R value (i.e.≥10%) were recollected. The final R values for each data set are listed in Table 1.

In this study, 5 different data sets were used, namely Parent (50mM glucose), AT2, Inosine, FMN and Caffeine. All of these were at 2.5 angstroms resolution. Most of the data for the Parent and Caffeine were collected by R.Fletterick and others(Sprang and Fletterick 1979).

Heavy atom derivatives, ethylmercurithiosalicylate(EMTS), dimercury acetate(DMA) and a "double-derivative" Pb-EMTS, have been used to extend the x-ray diffraction data set from 3.0 to 2.5 angstroms resolution in the Multiple Isomorphous Replacement (MIR) phasing procedure (Sprang and Fletterick 1979). In the DMA experiments caffeine was used as a stabilizing agent since crystals soaked in DMA alone showed an increased radiation sensitivity (from 6 to 7 % loss in diffracted intensity per hour at 3 angstroms resolution). Inclusion of caffeine at 10 mM in the soaking buffer reduced the radiation damage to 3% per hour. Caffeine showed up as the fouth highest peak in the difference electron density map and was used in the MIR phasing procedure.

The difference Fourier synthesis for each ligand binding experiment was calculated using the Delta F between protein complex and parent plus the most recent 2.5 angstrom native MIR phases. The programs used are DIFFER and FOURIER. The latter performs Fourier synthesis while the former calculates Delta F and scales data anisotropically. This is important for some poorly integrating data, but it is not important in this study because all the ligands promote the T state of the enzyme which is already present in this crystal form, so the data are isomorphic with respect to all the three axes X, Y and Z.

Graphics System (MMS-X)

The MMS-X (Molecular Modelling System) graphics system,

purchased from Washington University, St.Louis, was designed primarily for crystallographic model building, but at the time of this writing is a research tool under continual development and not a routine working instrument. It has a large number of built-in options. The system allows the three dimensional presentation and interactive manipulation of molecular structures (i.e. global orientation and translation of structures, and also those conformational changes that can be represented by bond rotations with up to six bonds.), together with background displays of contoured electron density maps. This instrument is meant to be a sophisticated version of the Richards' box discribed below.

Model fitting

Preliminary model fitting was done by contouring the different density maps and displaying them in the Richards' box (Richards 1968). Fig 1 represents a schematic drawing of the Richard box. Since the scale of the model is identical with that of the map, the corresponding positions in the map and in the model will appear superimposed when the model is fitted correctly, and they will not move with respect to each other regardless of the position of viewing. Labquip Models (1 cm/angstrom) were used and map sections were calculated for two orientations, one perpendicular to the crystallographic z-axis direction and the other perpendicular to the crystallographic x-axis direction.

Once the rough conformation of molecule has been found, it was used as a guide for choosing the most appropriate

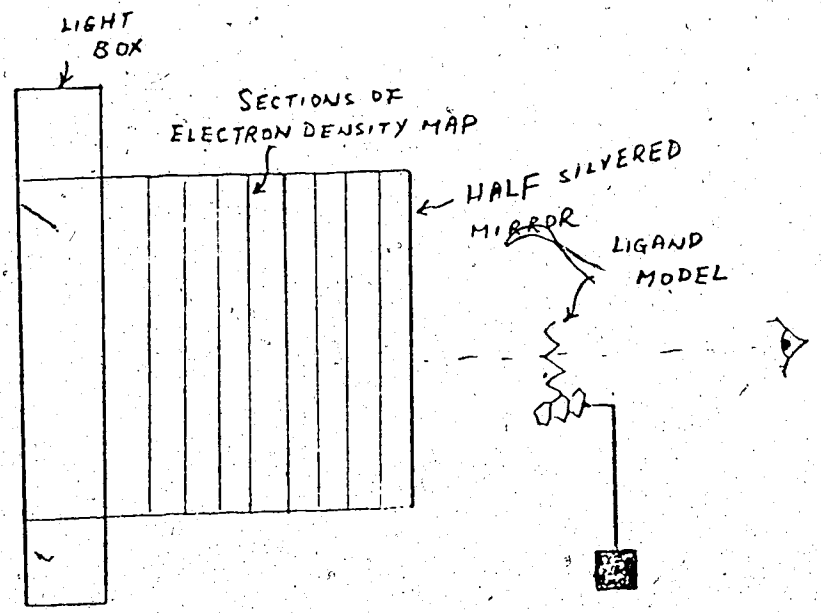


Fig. 1. Schematic drawing of the Richards box. (see text)

structure from published papers (of small molecules structures which best represented the ligand; Caffeine: Sutor 1958; Inosine: Thewalt et al 1970; FMN: Bear et al 1973; ATP: Kennard et al 1970), which is then fitted into the density map with the MMS-X system. Co-ordinates of the ligands were displayed on the screen. By doing single bond rotations, global rotation and translation, the models were fitted into the density map (which was contoured in

3-dimensions and appeared as a net form) to give the present co-ordinates. The contour level for these maps were arbitarily assigned to provide maximum interpretability before noise level gets too high as judged by number of peaks in solvent region.

Stereo-drawings of the fitted ligands were done by decoding the graphic display file generated by the MMS-X system and plottings were done by the University of Alberta Calcomp plotter.

Co-ordinates of the native proteins were obtained from the model building in this laboratory (Sprang and Fletterick 1979). These are unrefined and contain error of > 0.5 Angstroms.

Least squares planes through the atoms of the bases were calculated by the method of Blow(1960).

Computer programs

The authors of the computer programs employed in the present study and their functions are listed as followed.

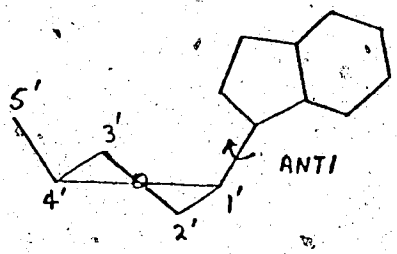
NAME	AUTHOR	FUNCTION
CRUNCHO	Jurgen Sygusch	Data reduction
CRYM	George Reeke	Data merging
DIFFER	David Blow/others	Delta F calculation, scaling
FCURIEP	George Reeke	Fourier synthesis
PL2	Robert Fletterick	Contour electron density map

MAPPREP	Stephen Sprang	Transfer electron density map from AMDAL to MMS-X
MMS-X	Washington U.	Graphics system
STEREO	Colin Broughton	Stereo plot of MMS-X graphic displays
ACCESS	T. J. Richmond	Calculate accessible surface area according to algorithm of Lee & Richards
GRADIENT	Sprang & Fletterick	Calculate electron density gradient at given co-ordinates

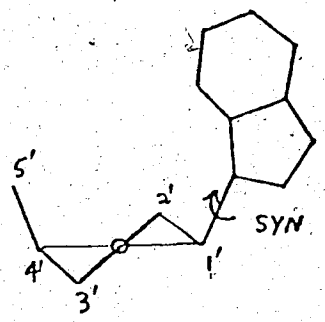
B. Results.

Nomenclature System

The nomenclature systems for the molecules studied are presented in Fig 2. Dihedral angles are named according to the system of Sundaralingam as illustrated also in Fig 2.



C(3') ENDO
C(2') EXO



C(2') ENDO
C(3') EXO

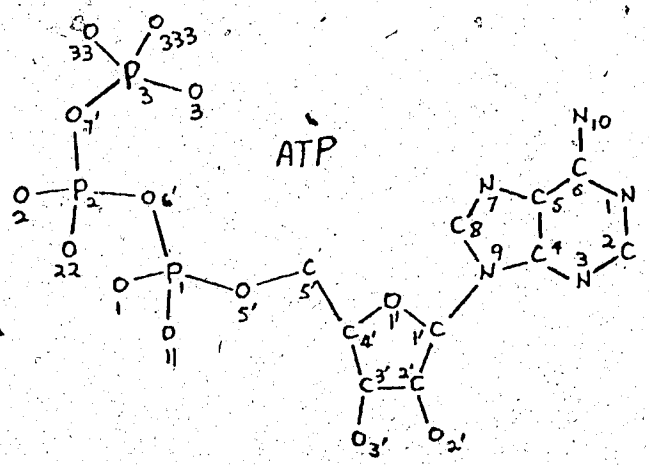
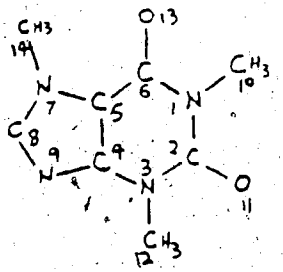
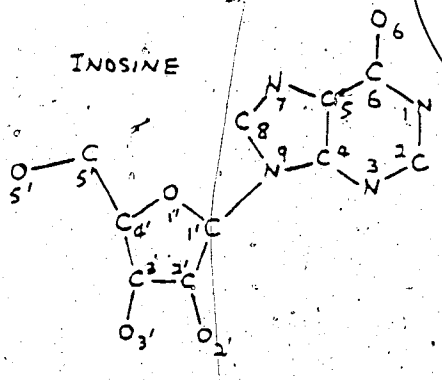


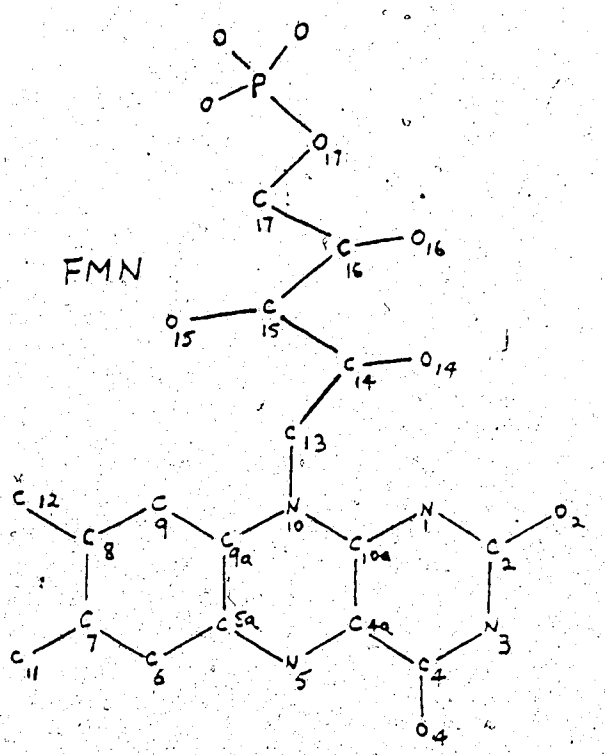
Fig. 2. Nomenclature system used for this thesis. The ribose is named according to its conformational state. If the C2' is deviated from the plane (made by atoms C4', C1' and O1') towards the same direction as the C5', the ribose will be called C2' endo. When the deviations of C2' and C3' from the plane are the same, the ribose can be called either endo or exo, whereas when the deviation is dorminated on one side, only one of the two name can be used which points to the atom with greater deviation.



CAFFEINE



INOSINE



FMN

Fig. 2. Continued.

Conformation of Molecules

a preliminary fitting was carried out because of the ease of obtaining the overall conformation by manual measurement compared to the MMS-X system, especially when dealing with a ribose ring as in Inosine and ATP etc. As mentioned in the Materials and Methods, in the preliminary model fitting, the maps were contoured twice in two orthogonal directions because it was impossible to fit the model unambiguously by looking from one direction only. Contour maps in the direction of X-axis placed underneath the model provides an additional guideline for the fitting. Then the appropriate structures were chosen from published papers as the initial structures for MMS-X which generated the present coordinates.

Fig 3(a-g) illustrate the best fit of the Caffeine, Inosine, FMN and ATP molecules to the difference electron density map as obtained by the procedure described above. The following is a description of the conformation of each individual molecule.

Caffeine:-

Since the map does not resolve individual atoms, the caffeine molecule is treated as a rigid body when fitted into the map. Contouring at a higher level of electron density should indicate the position of the ring. Considering this, we have obtained two possible

Fig 3(a-g). These figures represent the interpretation of the difference electron density map, the contour level for the map is arbitarily assigned as described in the Materials and Methods.

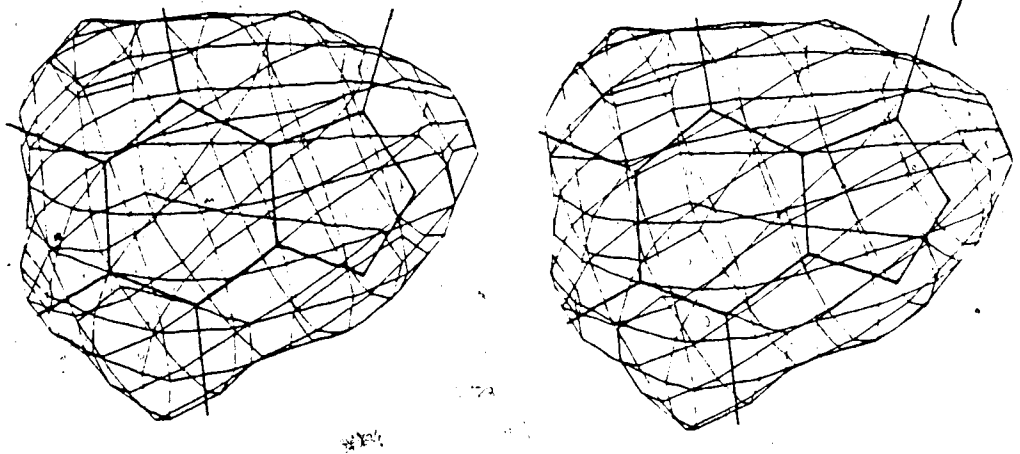


Fig. 3a. Caffeine 1

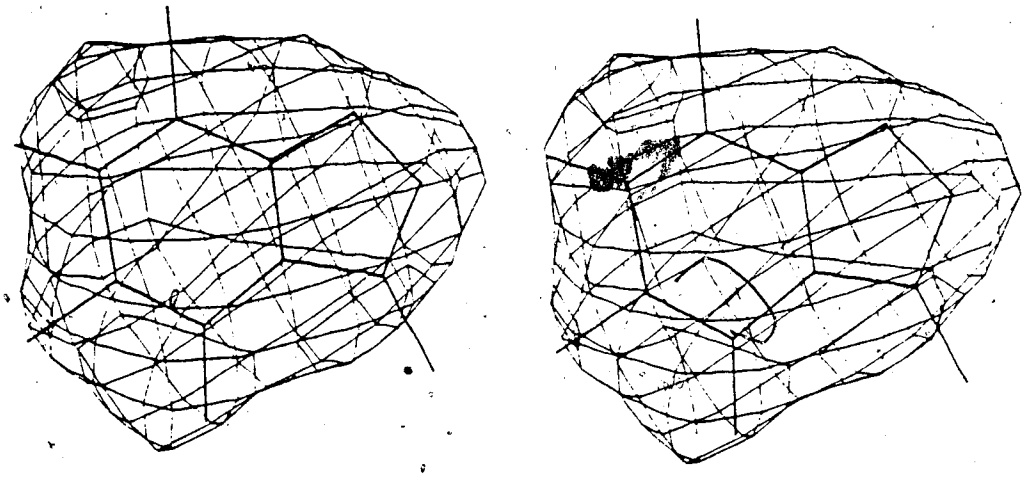


Fig. 3b. Caffeine 2

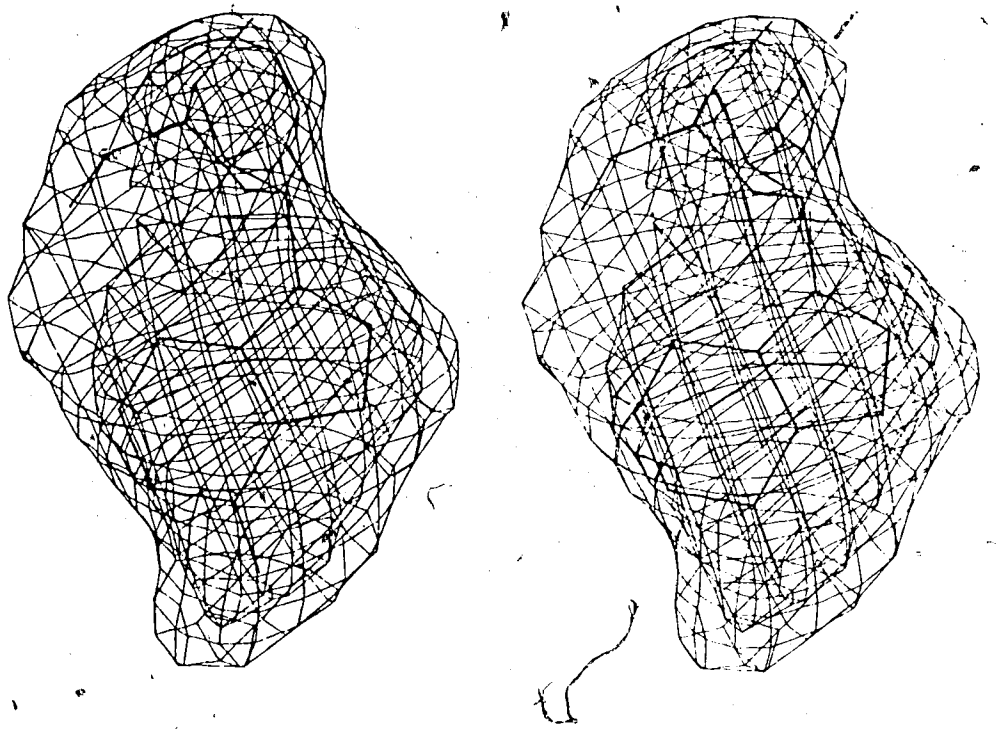


Fig. 3c. Inosine

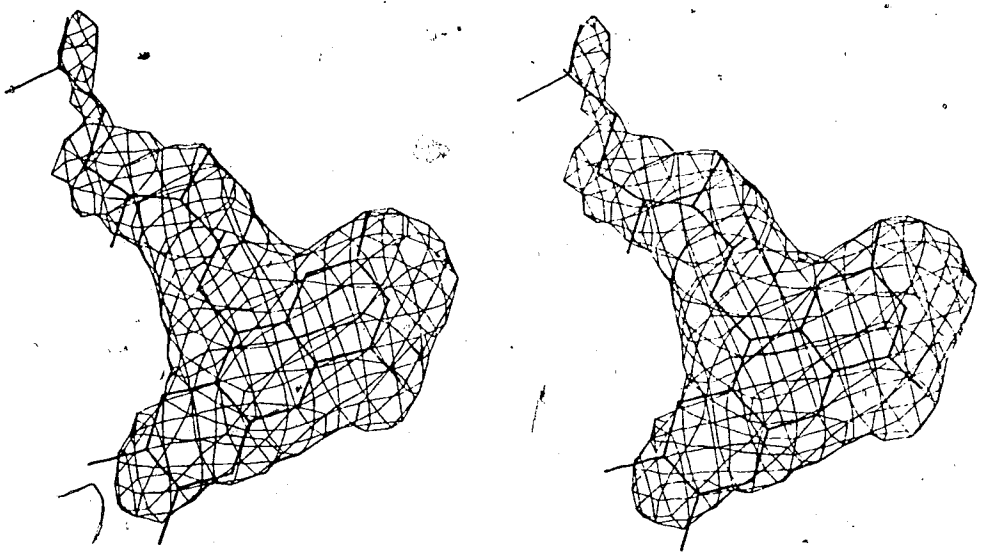


Fig. 3d. FMN

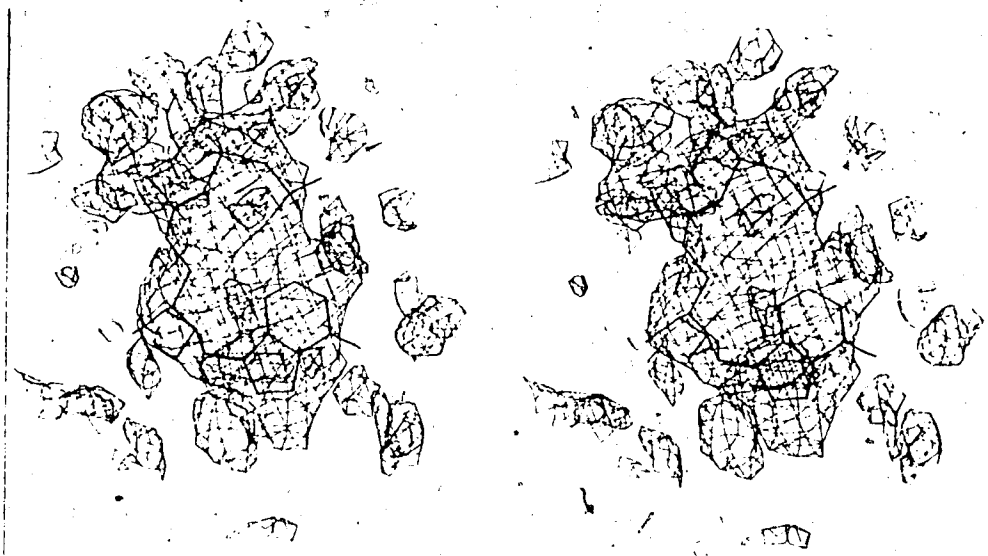


Fig. 3e. ATP (SYN)

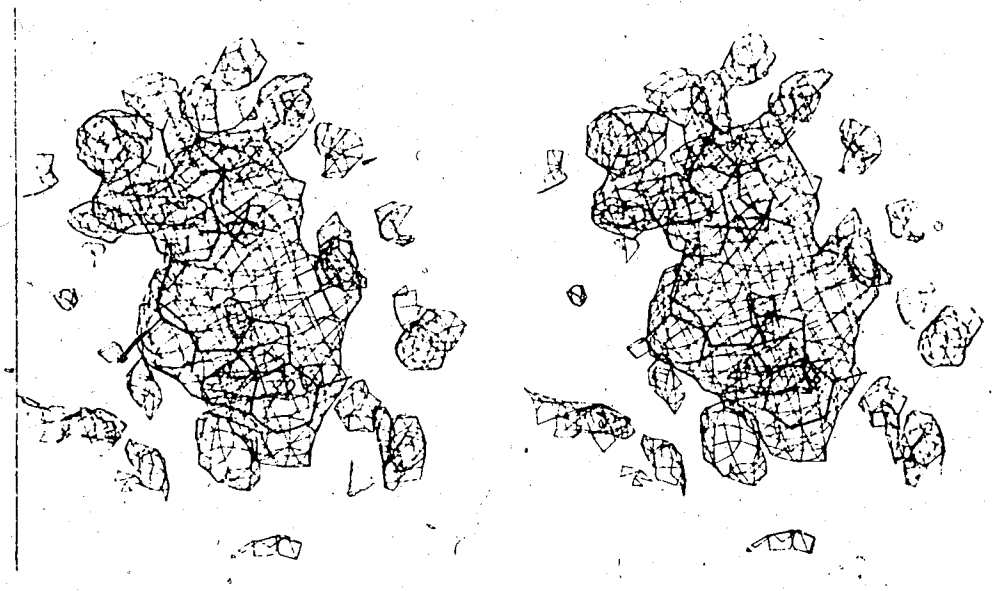


Fig. 3f. ATP (ANTI)

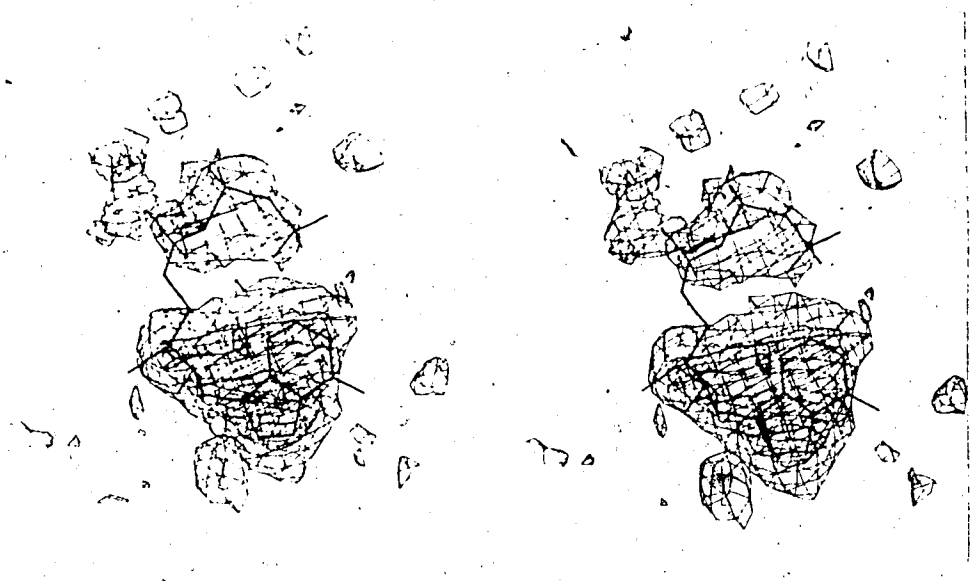


Fig. 3g. ATP in the SYN conformation with a higher contour level of electron density.

orientations of the molecule which correspond to a 180 degree rotation along the length of the molecule. It is difficult to conclude at this point whether one of the two or both was the preferable oreintation based on the present density map. Further discussion will be presented in latter section.

Inosine:-

During the preliminary studies, it was discovered that the inosine molecule exists in the SYN-conformation which is different from the preferred ANTI-conformation in solution as published (Schweyer et al 1968; Danyluk and Hruska 1968). In this study, only the C(2') endo and C(3') endo-puckering of the ribose have been considered. since these are known to be the most stable forms

(Sundaralingam 1969). From observation in the Richards box, it is found that only the C(2') endo-puckering of the ribose ring gives a reasonable fit.

The dihedral angle CHI of the molecule is 232 degrees, which falls within the boundary proposed by Sundaralingam. This will be discussed in more detail in the next section.

The distance between the O(5') of the ribose and the N(3) of the base is found to be 2.6 angstroms. Therefore the formation of hydrogen bond between these two atoms which stabilizes the SYN-conformation is highly likely.

FMN:-

Different conformations of the rings have been observed for the FMN molecule (Bear et al 1973; Smith et al 1977). Since no conclusion could be drawn on the planarity of the molecule at this resolution level, the molecule was taken as coplanar in this study.

Considering the fact that oxygen is more electron dense than the methyl group, the part of the map bearing higher electron density is assigned to the oxygen-bearing ring. In building the ribityl tail of the molecule, it was possible to arrange all the atoms in a staggered position and have them fitted reasonably well into the electron density map at the same time. The

isoalloxazine ring can be fitted with a moderate degree of flexibility. However, due to the presence of the ribityl tail this flexibility is restricted. The density map also allows a limited degree of freedom for the ribityl tail, but these variations are eliminated if the dihedral angles are considered to be restricted in a staggered position.

The portion of the density map corresponding to the phosphate group is not well defined. This may be due to the possibility that the phosphate group which lies at the solvent region of the protein is freely rotating.

Table 2 is a list of the dihedral angles of FMN, ATP and Inosine.

ATP:-

Fitting of the ATP molecule into the difference density map of 2.5 resolution posed a rather difficult problem due to the large degree of freedom of the ATP molecule which includes the large number of dihedral angles and the different puckering of the ribose ring. Since the C(2') endo and the C(3') endo are the most stable conformation, only these two types of sugar conformers have been considered in the fitting procedure. When the difference density map was contoured at the level regularly used for the study of other ligands, it was observed that the contour map could not accomodate the

Table 2
Dihedral angles of FMN, Inosine and ATP.

ATP	χ	ψ	ϕ	ω_1	ω_1'	ω_2	ω_2'	ω_3
	246	-174	130	59	160	145	-32	114

Inosine	χ	ψ
	232	-75

FMN	χ_1	χ_2	χ_3	χ_4	χ_5	χ_6	χ_7
	25	176	67	160	63	164	96

whole molecule. Apparently part of the molecule was not resolved at this contour level. Therefore, another contour map was produced at a much lower level to accomodate the whole molecule. Taking into consideration that although the phosphates should be more electron dense than the rest of the molecule, they are likely to be moving around in the solvent region, then the resulting density would be lower than that of the ribose ring plus the base. Therefore the denser region was assigned to the ribose ring and the base. Both the C(2') endo and C(3') endo have been fitted in the SYN and ANTI conformation. It was clear that the C(2') endo-SYN conformation can be best fitted. However, the possibility of having an ANTI conformer cannot be excluded. Since ATP strongly favors the ANTI conformation (Berthod and Pullman 1973), a fitting of the map with an ANTI ATP is also included for the reader to compare the two fittings. The phosphates can be fitted in a number of positions related by rotations

along different diphosphate bonds as shown in fig3e. As the contouring was done at an intermediate level, only one of the five conformations was observable which was therefore taken as the most preferable position (fig 3(e,f,g).

In studying the relative conformations of the bases of these ligands with respect to the protein, it was observed that there were two amino acid residues in the neighbourhood of the ligands which sandwich the base in a stacking fashion. They are the Tyrosine 6/12 and the Phenylalanine 285. Fig 4(a-e) illustrate the stacking positions for Caffeine, Inosine, FMN and ATP. They are all found to be bound to the protein in a relatively coplanar manner except FMN, which shows a deviation of about 25 degrees which will be accounted for in the later section. Moreover all these ligands adopt a more or less parallel longitudinal axis when stacked (in other words, if the direction along the length of each molecule is defined as its longitudinal axis, the axes of different ligands adopt a similar orientation). Table 3 gives the equation of planes of the various ligands. The angles between these planes have also been computed and listed in Table 4. Co-ordinates for ligands are listed in Table 5(a-e).

Protein - Ligand Interactions

To investigate the overall structural changes of the

Fig. 4(a-e) are illustrations of how the ligands are stacked between the two amino acids Phe-285 and Tyr-612, all the ligands are being projected on the plane parallel to the ring of Tyrosine 612.

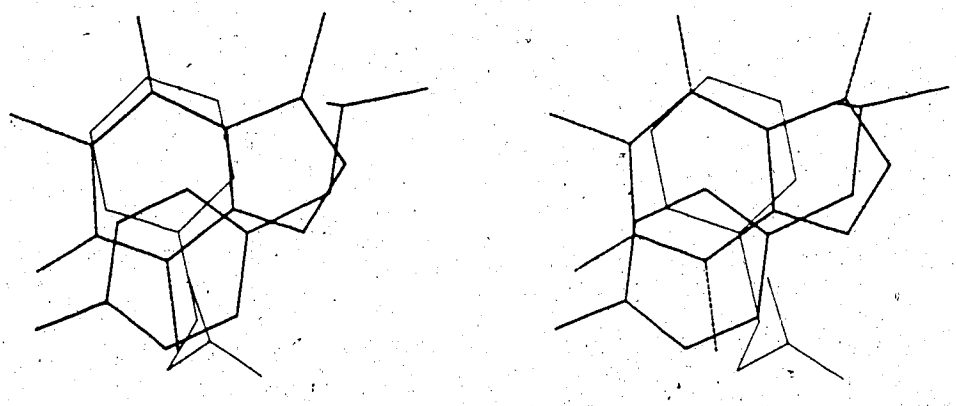


Fig. 4a. Caffeine 1

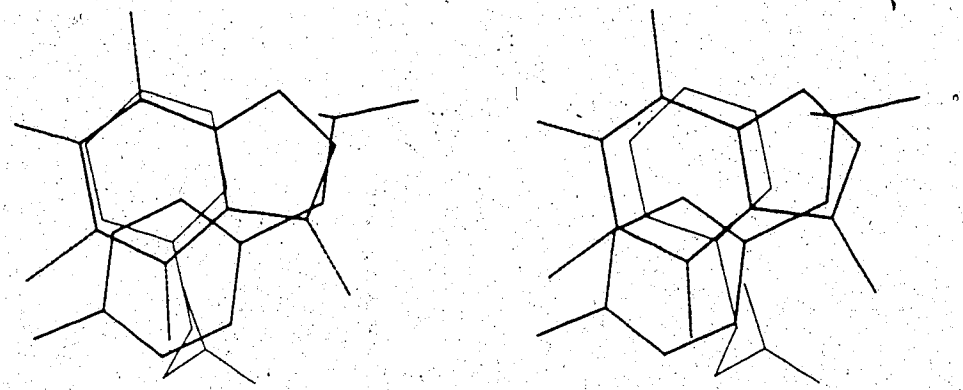


Fig. 4b. Caffeine 2

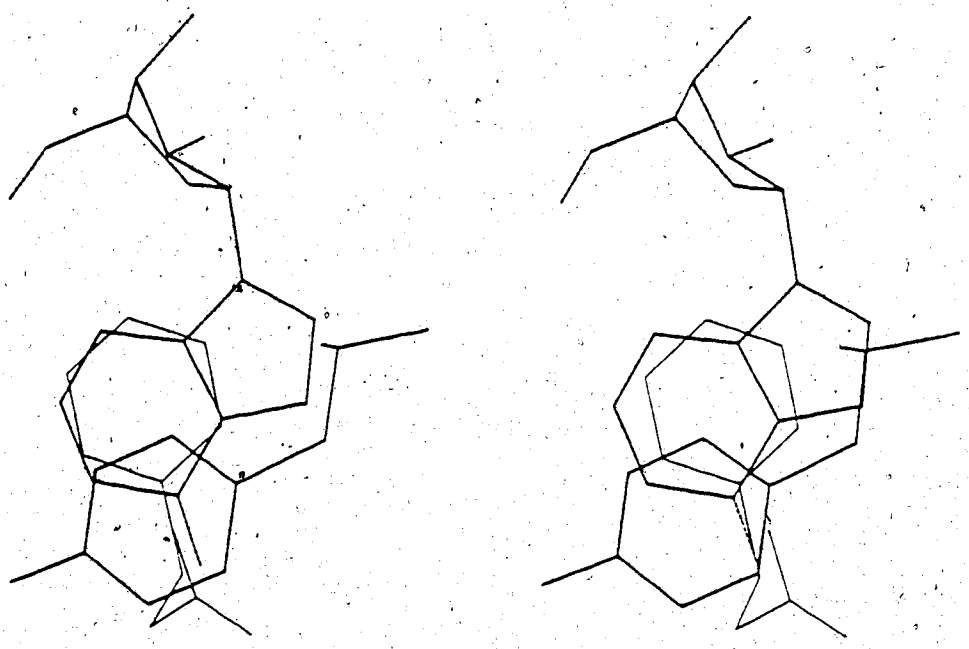


Fig. 4c. Inosine

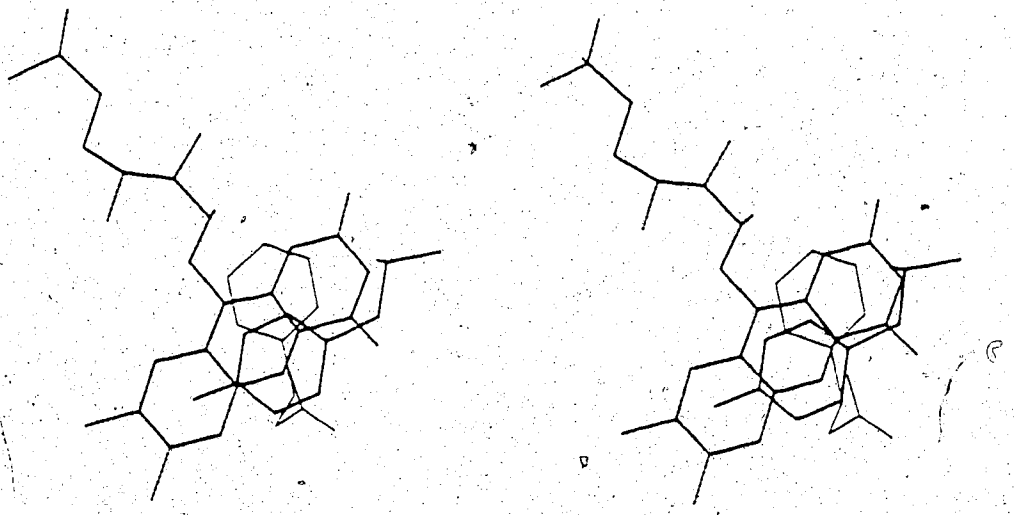


Fig. 4d. FMN

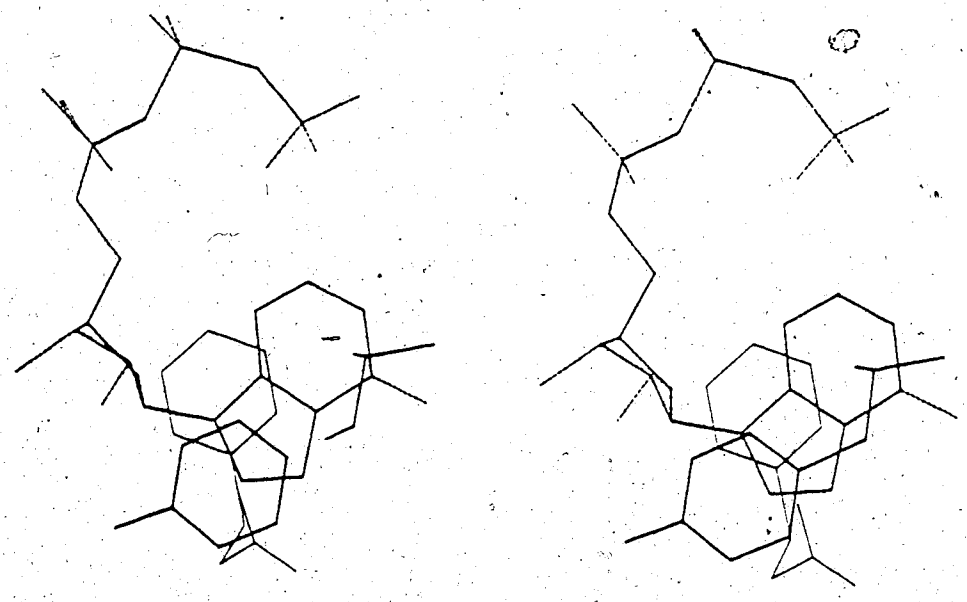


Fig. 4e. ATP

TABLE 3

Equaticns of least square planes of the ligands and the two stacking amino acids, XCosA+YCosB+ZCosC=D, where D = perpendicular distance frcm the origin of the unit cell to the plane where all the atoms of the ring lie. A, B and C are angles between the normal to the plane and positive X, Y, Z axes.

NAME	CosA	CosB	CosC	D
Phe-285	-0.619	0.655	-0.433	-16.53
Tyr-612	-0.709	0.623	-0.329	-10.98
Caffeine1	-0.675	0.687	-0.269	- 8.88
Caffeine2	-0.693	0.653	-0.307	-11.38
Inosine	-0.794	0.545	-0.270	-18.72
FMN	-0.892	0.313	-0.328	-32.45

TABLE 4

Dihedral angles between the least squared planes of table 2.

NAME	Phe	Tyr	Caff1	Caff2	Ino	FMN	ATP
ATP	12.6	7.6	2.6	5.4	11.4	26.2	0.0
FMN	25.9	20.7	25.2	22.8	14.9	0.0	
Ino	15.1	7.4	10.6	8.7	0.0		
Caff2	8.4	2.3	3.1	0.0			
Caff1	10.1	5.4	0.0				
Tyr	8.8	0.0					
Phe	0.0						

Table 5(a-e) are lists of Cartesian coordinates of the fitted ligands defined in the unit cell with the standard origin as used for the protein. The three columns corresponds to the x, y and z coordinates in Angstroms.

TABLE 5a. Caffeine1

	X	Y	Z
N1	38.77	36.64	29.40
C2	37.85	35.56	28.92
C10	39.70	37.14	28.36
C6	38.79	37.18	30.65
N3	37.03	35.15	29.84
O11	37.92	35.26	27.77
C12	36.08	34.06	29.46
C4	36.99	35.67	31.17
C5	37.84	36.64	31.57
N9	36.27	35.22	32.17
O13	39.64	38.10	30.90
N7	37.53	36.90	32.92
C8	36.60	35.99	33.22
C14	38.19	37.86	33.82

Table 5b. Caffeine2

	X	Y	Z
N1	37.96	35.75	29.06
C2	38.91	36.89	29.34
C10	38.05	35.22	27.69
C6	37.08	35.21	29.95
N3	38.82	37.32	30.56
O11	39.66	37.18	28.46
C12	39.72	38.46	30.93
C4	37.93	36.81	31.54
C5	37.08	35.80	31.25
N9	37.72	37.28	32.74
O13	36.34	34.25	29.55
N7	36.36	35.54	32.43
C8	36.77	36.50	33.28
C14	35.29	34.54	32.64

Table 5c. Inosine

	X	Y	Z
N1	37.90	35.38	29.32
C2	38.71	36.47	29.20
C6	37.23	35.01	30.51
N3	38.94	37.36	30.12
C4	38.35	37.03	31.31
C5	37.51	35.94	31.54
O6	36.56	34.01	30.56
N7	37.06	35.96	32.86
N9	38.45	37.68	32.49
C8	37.65	37.02	33.37
C1'	39.28	38.88	32.74
O1'	38.87	39.77	31.67
C2'	40.70	38.60	32.53
C4'	40.05	40.52	31.26
O2'	41.28	38.02	33.65
C3'	41.15	40.02	32.19
O3'	41.20	40.82	33.33
C5'	40.20	40.41	29.81
O5'	40.68	39.12	29.46

Table 5d. FMN

	X	Y	Z
N01	38.19	37.65	31.03
N03	37.19	36.62	32.94
O02	37.97	38.93	32.72
C02	37.75	37.81	32.27
C04	37.00	35.47	32.27
O04	36.45	34.55	32.87
C04A	37.45	35.29	30.88
N05	37.31	34.20	30.31
C10A	38.03	36.47	30.31
N10	38.50	36.35	29.09
C05A	37.65	34.07	29.05
C06	37.43	32.80	28.40
C09A	38.31	35.16	28.39
C09	38.80	34.98	27.09
C13	39.27	37.55	28.40
C07	37.83	32.70	27.04
C08	38.52	33.71	26.44
C12	38.97	33.55	24.97
C11	37.59	31.35	26.34
C14	39.77	38.27	29.37
O14	40.71	37.51	30.20
C15	40.59	39.38	28.69
O15	40.84	40.38	29.73
C16	41.85	38.96	27.92
O16	41.59	37.75	27.26
C17	42.37	39.96	26.89
C17	42.76	41.18	27.68
P17	43.70	42.23	26.88
O18	42.85	43.31	26.30
O19	44.77	42.59	27.85
O20	44.40	41.39	25.74

Table 5e. ATP (SYN)

	X	Y	Z
C2	38.72	37.86	33.00
N1	37.88	37.26	33.91
N3	38.80	37.57	31.73
C4	37.94	36.58	31.38
C5	36.97	35.96	32.17
N9	37.81	36.03	30.12
C6	36.93	36.33	33.55
N10	36.12	35.81	34.43
N7	36.30	35.00	31.45
C8	36.79	35.07	30.22
C1'	38.78	36.16	28.96
C2'	38.75	37.63	28.51
O1'	39.96	35.84	29.59
C4'	41.06	36.71	28.97
O2'	38.00	37.52	27.27
C3'	40.32	37.48	28.11
O3'	40.47	36.86	26.72
C5'	41.67	37.48	30.17
O5'	42.73	38.55	29.67
P1	42.75	39.88	30.04
O1	41.36	40.46	29.61
O11	44.00	40.70	29.53
O6'	42.88	39.86	31.47
P2	43.38	40.94	32.66
O2	44.92	40.48	32.92
O22	43.13	42.27	32.08
O7'	42.35	40.55	33.95
P3	40.92	40.04	34.18
O3	41.27	38.68	34.81
O33	40.25	40.83	35.15
O333	40.32	39.84	32.90

protein induced by these ligands, the gradient search program was used as described in Materials and Methods. This program calculates the electron density gradient at each of the atomic coordinates of the protein, the gradient will be greater than zero when there are structural changes. Therefore it can be used to locate the major structural changes within a protein if there are any. All the ligands have been examined except caffeine, due to the high noise level of the density map as mentioned previously. No significant main chain movement was observed, which agrees with the results of visual inspection of the density maps and the small value obtained from the % structure amplitude changes as listed in Table 1.

In checking for the side chain movements, only the area within six angstroms from the surface of the ligand was studied. No significant density mass was observed in the neighbourhood of the ligands, so it can be interpreted as absence of side chain movement in binding these ligands.

The inhibitor site contains twelve non-polar uncharged amino acids (Pro281, Phe285, Phe286, Leu379, Pro380, Ala382, Leu383, Pro610, Met614, Ala615, Ile760 and Met763), Six polar uncharged amino acids (Asn133, Asn282, Asn284, Tyr572, Gly611 and Tyr612), six basic amino acids (Arg385, His570, Lys573, His613, His767 and Arg769) and six acidic amino acids (Asp283, Glu287, Glu381, Glu384, Glu571 and Asp759) in a box within 4 angstroms surrounding the ligands (The box is defined by the minimum and maximum x, y, z coordinates of

the ligands plus an extension of four angstroms along each of the three axes in both directions. It appears as a rectangular box). Most of these amino acids are labelled in Fig 5e.

Table6a&b are lists of the distances between the ligands and protein side chain atoms of interest (those atoms pairs which may form hydrogen bond or electrostatic interaction). When the box dimension is extended to ten angstroms, the number of negative charged amino acids become slightly larger than that of positive charged amino acids. A number of hydrogen bonds may exist between ATP and the protein, however, due to the uncertainty of the atomic coordinates in this model plus the comparatively lower quality of the fitting of ATP, a firm conclusion has not been drawn at this point. Some posible pairs are N10 & ND of Asn282, O3' & OE2 of Glu381 and O2' & OE1 of Glu571. It is interesting that there are no short contacts (less than four angstroms) between the protein and the phosphates of ATP. However, the are four glutamic acids and one arginine in a box of 10 angstroms around the O5' of inosine. Table6a shows that besides the stacking amino acids Phe285 and Tyr612, the ligand only comes into contact with Asn282, glu287, His570, Glu571 and Gly611.

As mentioned before, all the ligands are sandwiched between Tyr-612 and Phe-285. An attempt has been made to investigate if these two amino acids have been tilted due to the inclined stacking of the isoalloxazine ring of FMN. No

TABLE 6a.

List of all van der Waals contacts between the ligands and Protein within four angstroms.

CAFFEINE 1		DISTANCE
HIS	570	
C12	CE1	4.0
GLY	611	
N7	O	3.5
C8	O	3.9
C14	C	3.8
C14	O	2.9
CAFFEINE 2		
ASN	282	
C14	ND	3.5
GLY	611	
N9	O	3.4
C8	C	3.8
C8	O	3.4
INOSINE		
GLY	611	
N9	O	3.7
C8	C	3.9
C8	O	3.2
C11	O	3.8
FMN		
ASN	282	
O04	ND	3.3
GLY	611	
N03	O	3.6
O02	C	3.6
O02	O	2.8
C02	O	3.4
ATP		
ASN	282	
N10	ND	3.2
GLU	287	
N1	OE2	3.9
N10	OE2	3.8
GLU	381	
O3'	OE2	3.5
GLY	611	
C2	O	3.6
N1	C	3.9
N1	O	3.0
C6	C	4.0
C6	O	3.5
N10	O	3.8
GLU	571	
O2'	OE1	3.2

TABLE 6b.

List of all the interesting interactions between the protein and the ribose plus phosphates (that is possible hydrogen bonding pairs and electrostatic interacting pairs within ten angstroms).

INOSINE		DISTANCE
ASN	282	
O1'	OD	9.9
O1'	ND	8.5
O2'	OD	8.4
O2'	ND	7.7
O3'	ND	9.8
GLU	287	
O1'	OE1	9.1
O1'	OE2	7.1
O2'	OE1	6.9
O2'	OE2	5.1
O3'	OE1	8.8
O3'	OE2	7.2
O5'	OE2	8.8
LYS	289	
O2'	NZ	9.3
GLU	381	
O1'	OE2	9.4
O5'	OE1	8.4
O5'	OE2	6.8
GLU	571	
O1'	OE1	7.4
O1'	OE2	9.4
O3'	OE1	9.9
O5'	OE1	6.2
O5'	OE1	7.9
ASP	768	
O5'	OD2	9.9
ARG	769	
O5'	NE	9.7
O5'	NH2	7.8
O5'	NH1	8.1
FMN		
ASN	282	
O04	ND	3.3
O14	OD	9.7
O14	ND	8.6
GLU	287	
O14	OE1	9.7
O14	OE2	7.6
O15	OE2	9.3
GLU	381	
O14	OE1	8.0
O14	OE2	6.7
O15	OE1	9.4
O15	OE2	7.7

O16	OE1	5.7
O16	OE2	4.1
O17	CE1	8.7
O17	OE2	6.9
P17	OE1	9.3
P17	OE2	7.6
O18	OE2	8.2
O19	OE2	8.6
O20	OE1	8.3
O20	OE2	6.7
GLU	571	
O14	OE1	7.0
O14	OE2	8.8
O15	OE1	6.7
O15	OE2	8.3
O16	OE1	5.5
O16	OE2	6.8
O17	OE1	7.0
O17	OE2	7.9
P17	OE1	7.9
P17	OE2	8.5
O18	OE1	7.6
O18	OE2	8.0
O19	OE1	9.3
O19	OE2	9.9
O20	OE1	8.0
O20	OE2	8.3
ASP	759	
P17	OD1	9.9
O18	OD1	8.5
O18	OD2	8.9
ASP	768	
O16	OD1	9.3
O16	OD2	7.8
O17	OD1	8.7
O17	OD2	8.0
P17	OD1	7.9
P17	OD2	7.5
O18	OD1	7.6
O18	OD2	7.4
O19	OD1	9.0
O19	OD2	8.7
O20	OD1	6.8
O20	OD2	6.3
ASP	769	
O18	NE	9.4
O18	NH2	8.6
O18	NH1	7.4
O19	NH2	9.3
O19	NH1	8.2
O20	NE	8.0
O20	NH2	7.1
O20	NH1	5.8

ATP		
ASN	282	
N10	ND	3.2
O1'	OD	9.0
O1'	ND	7.9
C2'	ND	9.8
P3	OD	9.8
P3	ND	8.9
O3	OD	8.6
O3	ND	7.9
O33	ND	9.1
O333	OD	9.8
O333	ND	8.7
GLU	287	
O1'	OE1	9.8
O1'	OE2	7.7
O5'	OE2	9.1
P1	OE2	9.4
O1	OE2	9.5
O6'	OE2	8.4
P2	OE2	8.6
O2	OE2	9.2
O22	OE2	9.7
O7'	OE1	8.7
O7'	OE2	7.2
P3	OE1	7.7
P3	OE2	6.1
O3	OE1	6.5
O3	OE2	4.9
O33	OE1	7.5
O33	OE2	6.1
O333	OE1	8.3
O333	OE2	6.5
N1	OE2	3.9
N10	OE2	3.8
LYS	289	
O7'	NZ	9.6
GLU	381	
O1'	OE1	7.1
O1'	OE2	6.0
O2'	OE1	7.3
O2'	OE2	5.6
O3'	OE1	5.2
O3'	OE2	3.5
O5'	OE1	7.9
O5'	OE2	6.6
P1	OE1	9.0
P1	OE2	7.6
C1	OE1	9.3
O1	OE2	7.6
O11	OE1	9.4
O11	OE2	7.9
O6'	OE2	8.8

GLU	571	
O1'	OE1	6.6
O1'	OE2	8.5
O2'	OE1	3.2
O2'	OE2	5.3
O3'	OE1	4.7
O3'	OE2	6.0
O5'	OE1	7.8
O5'	OE2	9.2
P1	OE1	8.1
P1	OE2	9.5
O1	OE1	6.9
O1	OE2	8.4
O11	OE1	8.9
O6'	OE1	9.2
O33	OE1	9.1
ASP	768	
O2'	OD2	9.2
O3'	OD1	9.5
O3'	OD2	7.8
O5'	OD2	9.9
O1	OD2	9.9
O11	OD2	9.8
ARG	769	
O1'	NE	9.5
O1'	NH2	7.3
O1'	NH1	8.4
O2'	NE	8.1
O2'	NH2	6.2
O2'	NH1	7.3
O3'	NE	6.6
O3'	NH2	4.5
O3'	NH1	5.4
O5'	NE	9.7
O5'	NH2	7.8
O5'	NH1	7.9
P1	NH2	8.7
P1	NH1	8.6
O1	NH2	8.6
O1	NH1	8.5
O11	NH2	9.0
O11	NH1	8.4
O6'	NH2	9.9
O6'	NH1	9.9

TABLE 7

Surface area buried and the energy involved (see text). Due to limitation of space abbreviations are used for this table. When the name of the ligand first appears it is given the full name, XXX-C = ligands - protein complex, XXX+C = ligands plus protein. XXX-C is a measured quantity while XXX+C is a calculated quantity. Delta Gh = hydrophobic free energy, Delta Gtr = translational and rotational free energy, Delta Gd = free energy of dissociation and Delta H = enthalpy of the interaction.

Name	Surface area	Change in area	T Δ S ΔGh	ΔGtr	ΔGd	ΔH
Protein	4536.8					
Caffeine1	348.8					
Caff1+C	4885.6					
Caff1-C	4409.6	476.0	12.0	-18	-5.5	-11.5
Caffeine2	351.3					
Caff2+C	4888.1					
Caff2-C	4409.7	478.4	12.0	-18	-5.5	-11.5
FMN	618.6					
FMN+C	5155.4					
FMN-C	4501.8	653.6	16.3	-20	-6.8	-10.5
Inosine	382.7					
Ino+C	4919.5					
Ino-C	4508.9	410.6	10.3	-18.5	-4.0	-12.2

conclusion could be drawn due to the ambiguity of the map.

Surface area calculation

To understand and interpret the ligand - protein interaction is very difficult. Recently Janin and Chothia attempted to explain this problem by using the concept of hydrophobicity (Janin and Chothia 1978). Changes in accessible surface area as defined by Lee and Richards (1971) had been used to determined the degree of hydrophobicity change. Using the same concept the surface area changes of the ligands before and after binding to the protein have been calculated and listed in Table 7. This will be described below in more detail.

Energy for the Binding of Ligands

Taking the empirical value of 25 cal per square angstrom of accessible surface area buried (Chothia 1974), the hydrophobic free energy has been calculated as listed in Table 7. The number of 25 has been derived from experiments on the solubilities of amino acids in different solvents by measuring the free energy of transfer of amino acid from a non-polar reference solvent tc an aqueous medium. Fig 5&6 are space filling and stick-bond drawings which provide a better visualization of the actual ligand-protein interaction.

C. Discussion.

MMS-X System

Since MMS-X has been used throughout the study for the interpretation of the results, it is desirable at this point to comment cn scme of the features that make it superior to the Richards Box system. Firstly, since both the molecule and the density map can be rotated freely and can be observed from any orientation desired, it gives a much better fit than the Richards box. Secondly, since the system produces the measurement of ccordinates autcmatically, this avoids the random errors generated by manual measurement as well as the systematic error due to the Richard box such as

Figures 5 & 6(a-d) are stick-bond and space filling drawings of the four ligands being studied, the orientation of the ligands are all the same.

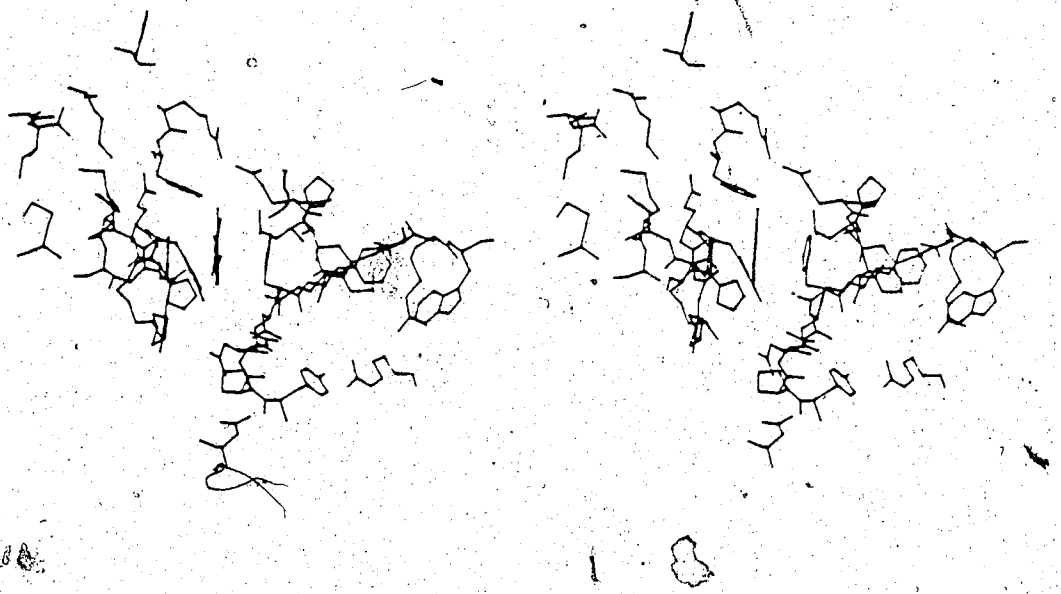


Fig. 5a. Caffeine

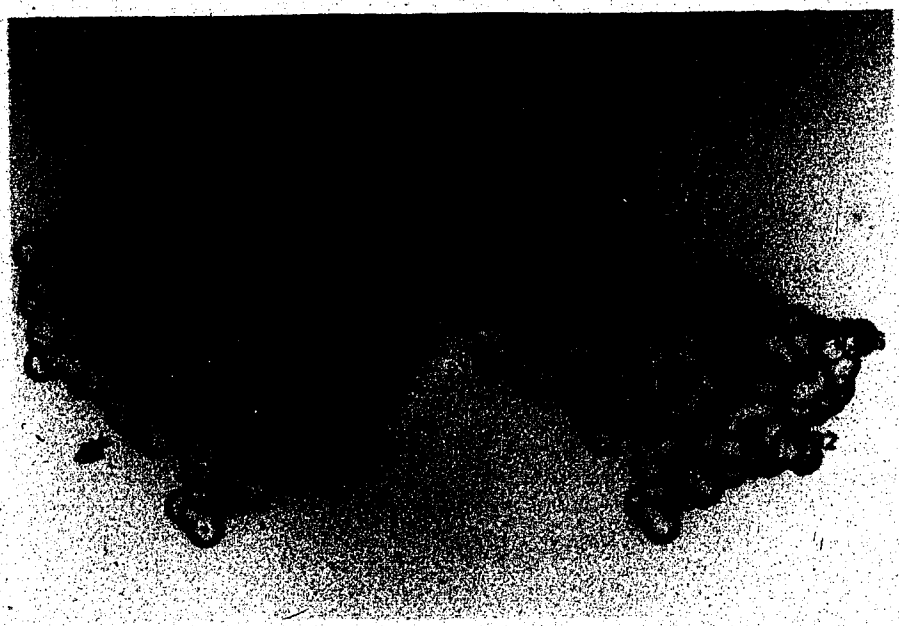


Fig. 6a. Caffeine

Fig. 6b. Inosine

Fig. 6b. Inosine

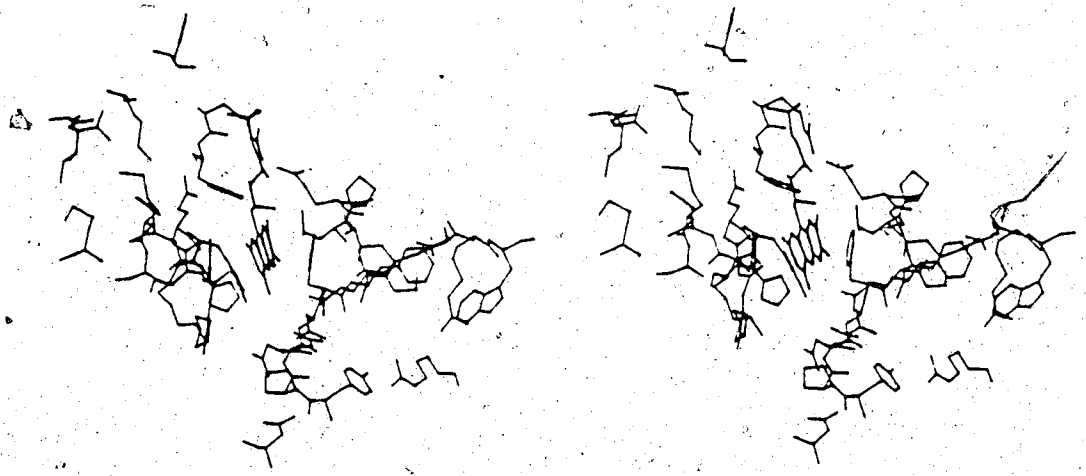


Fig. 5c. FMN

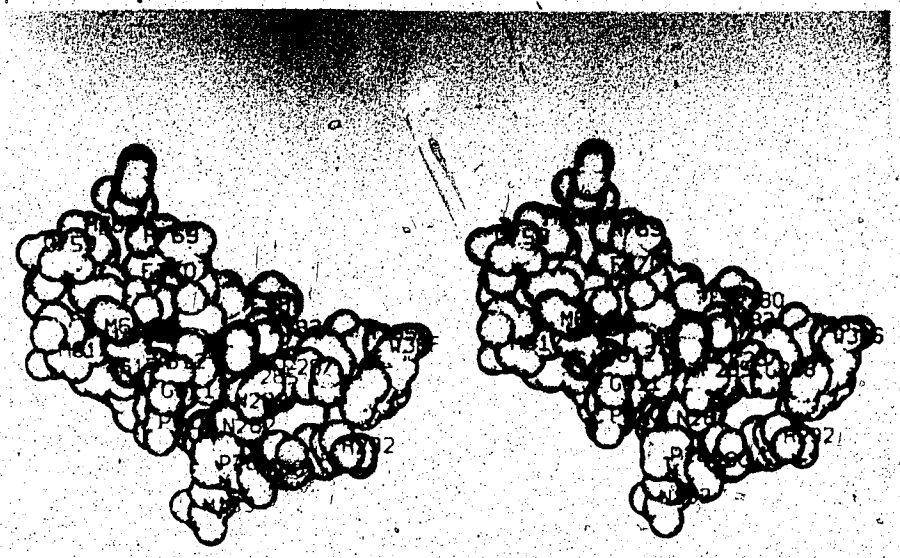


Fig. 6c. FMN

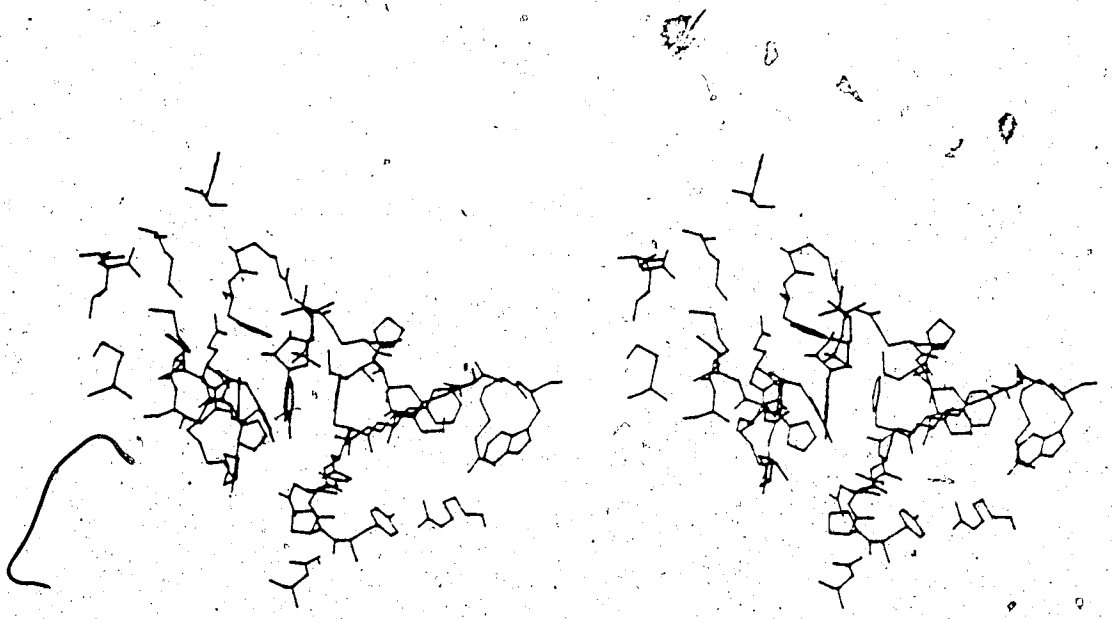


Fig. 5d. ATP (SYN)

Fig. 6d. ATP (SYN)

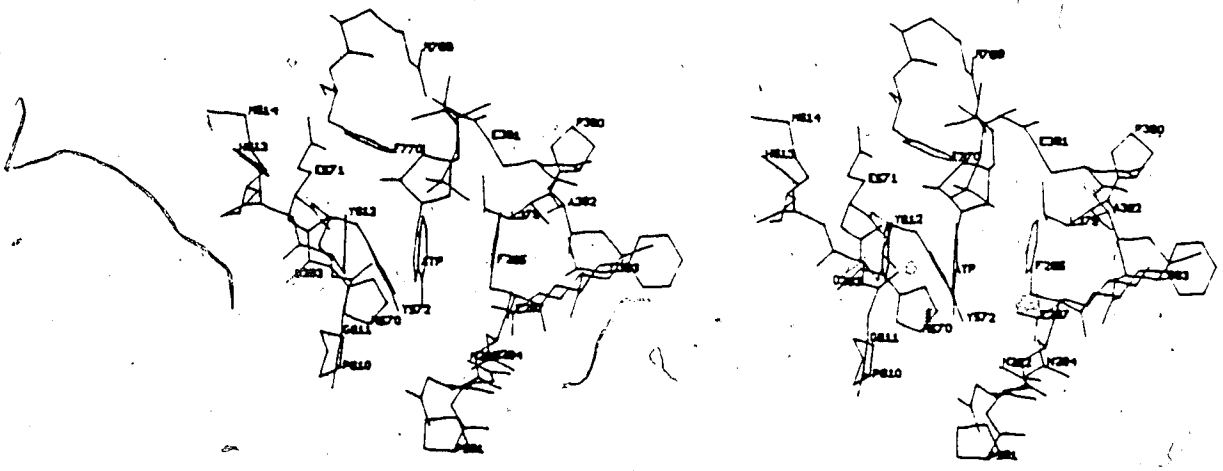


Fig. 5e. ATP (SYN). All amino acids are labelled with one letter code.

the diffraction (contraction) of the half-way mirror and the photo distortion due to xeroxing of the contour maps. Thirdly, the contour level of the map can be changed freely and figures can be prepared more easily and fitting can be criticized more readily.

Stacking interaction.

The stacking of the ligands between the Tyr-612 and Phe-285, is a complex π π (pi-pi), van der Waals interaction. With the exception of some electrostatic interactions with separation in the order of nine angstroms, no hydrogen bonds, charge or salt bridges have been observed, though hydrogen bonding might be suspected between the ring nitrogen of Histidine-570 and O6 of Inosine.

However, since the distance between these two atoms is 4.9 angstroms, the formation of hydrogen bonding is highly unlikely even allowing for the errors in the coordinates.

Since stacking is the only significant force stabilizing the interaction between the protein and ligands, it is expected that the degree of specificity of the binding is limited to the overall molecular structure of the ligand. This observation is consistent with the results of kinetic studies of rabbit muscle enzyme which show that the enzyme has relaxed specificity for a number of compounds of different ring structures ranging from purine analogues to isoalloxazine ring of riboflavin (Kasvinsky et al 1978b,c).

The present study is not the first incidence of co-enzyme stacked between two protein side chains. Similar binding has been reported of FMN in Flavodoxin (Watenpaugh et al 1973), in which case the stacking involved parallel stacking with a tyrosine and 45 degree inclined stacking with a tryptophan.

Stacking has been regarded as an important factor in stabilizing polynucleotide structures and in directing the binding of aromatic compounds to nucleic acids. In a review by Bugg, the base stacking pattern of over seventy crystal structures of nucleic acid constituents in polynucleotides had been examined (Bugg et al 1971). It was found that extensive overlapping between bases is unusual. Surprisingly, results from theoretical calculation also indicate that the highest geometric overlap between bases

does not necessarily lead to the most favorable stacking interaction (Orstein et al 1978). In the present study, as indicated by Fig. 4a-e, the ligands overlap extensively with Phe-285 but show only partial overlapping with Tyr-612. In the case of ATP, the adenine base does not penetrate deeply into the binding site. so the base ring is not stacked as extensively as the other ligands.

In an attempt to locate the stacking energy minima between bases, Orstein has calculated the potential energy of stacking as a function of the rotational angle and separation distance by decomposing the total base-pair stacking energy into its components, i.e. electrostatic, polarization, dispersion, and repulsion. It was observed that the electrostatic component controls the location of stacking minima, the most favorable relative base-pair orientation. Since the presence of heteroatoms in an aromatic hydrocarbon results in an asymmetric electrostatic environment, this is likely to control the mutual oreintation of the bases. As the number of heteroatoms and their location in the ring vary, in the absence of specific hydrogen bonding, the mutual orientation between the ligands and the stacking amino acid side chains differ with different ligands. In addition, the remaining parts of the molecule alsc impose a restriction on the orientation of the base, because they tend to position themselves in a relatively open area which is the solvent region.

As mentioned in the Results, the ligands were stacked

in a more or less parallel fashion with respect to Tyr-612 and Phe-285, with the exception of FMN, which exhibits a plane dihedral angle of 25 degrees. In the stacking of most bases, a plane dihedral angle of 0 - 5 degrees is quite common. The fact that the FMN has such a large inclinition may be accounted for by its increased size due to an additional ring which causes the molecule to tilt slightly in order to accomodate itself in the binding site. If it were not tilted, it would collide with His-570.

Conformation of nucleotides.

The glycosidic torsional angle CHI is used to indicate the relative orientation of the ribose with respect to the base in the nucleotide. It is defined by the rotation of the C8-N9 bond of the base about the N9-C1' bond with respect to the C1'-O1' bond of the sugar. The torsional angle is considered to be zero degrees when C8-N9 are cis-planar with respect to C1'-O1'. A positive CHI value is obtained as the base rotates in a clockwise direction when viewed along the C1-N9 axis. The ribose is in the ANTI conformation when CHI is equal to 0+/-90 and SYN when CHI is equal to 180+/-90 degrees (Fig. 2).

In the present study, it is observed that both ATP and inosine are most likely in the SYN conformation, with dihedral angles being 246 and 232, respectively. In a review by Sundaralingam investigating crystal structures of 25 nuclectides and nucleosides, he observed three distinct

regions for the CHI values of BETA Purine nucleoside, namely, 3-55, 120-123 and 210-258 degrees. In the last angular range, the conformation has been observed to be stabilized by O5-H...N3 hydrogen bonding, and all the riboses exhibit C(2') endopuckering (Sundaralingam 1969, 1975).

Our study with inosine, which exhibits similar characteristics would well fit into the last group. ATP, however, due to the absence of hydrogen bonding between O5 and N3, can not be compared with these groups of structures investigated. From another study by Sundaralingam it was also observed that nucleotides are conformationally more "rigid" than nucleosides (Rubin et al 1972). In addition, it was proposed that nucleotides exhibit only ANTI conformation about the glycosyl bond, while nucleosides may exist in both SYN or ANTI conformation. The applicability of the above generalization, however, would be limited by the size of the sample studied, which is restricted by the number of structures resolved by X-ray crystallography. Since it is questionable whether Sundaralingam's proposal, which was based entirely on the survey of X-ray crystallographic results (in solid state in crystals), represents a genuine fundamental difference with respect to the properties of the two groups of nucleo-compounds, a theoretical investigation which employs the PCILO computations has been performed by Pullman and associates (Berthod and Pullman 1973a,b). From the study of a number of purine and pyrimidine

5'-nucleotides, it was demonstrated that although guanosine ard inosine 5'-phosphates show a preference for the SYN conformation, most nuclectides prefer the ANTI conformation.

Pullman's results appear to lend some support to the previous proposal as well as to provide a better theoretical consideration of the problem. However, one has to be aware of the limitation of the methcdology of most theoretical calculations. In this case, the pseudo-rotation of the ribose has not been dealt with due to the complexity of the problem. The ribose was prefixed at either the C(2') endo or the C(3') endo conformaticns. Therefore the applicability of the results of the calculations is questionable.

Although nucleotides bound to enzymes are likely distorted frcm their preferred conformations in solution, the glycosyl torsion is mcst stubborn to distortion (Sundaralingam 1975). There has not been any report of 5' Adenine nucleotide with the SYN conformatior. In the present study the ATP was observed to be in the less preferred SYN conformation. The contradiction between this observation with the cited generalization can be accounted for by the following:-

1) The above gereralization may not be representative,
2) There might be some factors operating in this particular case which may give sufficient energy to overcome the glycosyl torsion energy barrier.

The first possibility has been considered in the above discussion. However, it is interesting to note that ATP when

bound to another site (AMP/ATP activator site) of the same enzyme also exhibits an ANTI conformation (Yang et al unpublished result 1978), which gives another incident to support the validity of the gereralization being discussed. Therefore, we have to go into the second suggestion to find a possible explanation of this unusual binding conformation. As has been pointed out, the molecules has to obtain sufficient energy a) to change from its "preferred" ANTI conformation in solution to the SYN conformation in the binding site, b) to have enough energy to stabilize the "high-energy" SYN conformation in the binding site. The second energy requirement can be easily met by the stacking energy generated after the ligand has been bound to the protein. The question now to be considered is therefore how the molecule undergoes its ANTI - SYN transformation. Let us consider a hypothetical situation in which ATP exists in the solvent in high molarity, there is finite probability that some molecules may have acquired sufficient energy for the ANTI - SYN transformation. Since only the SYN conformer can be fitted into the density map, it is likely that that the enzyme can stabilize this less stable conformation. As has been pointed out, this is possible only in the case of a high concentration of ligands.

The above idea has been corroborated experimentally. Kinetic studies carried cut by Kasvinsky et al (1978b,c) demonstrated that with adenine and hypoxanthine, the addition of a ribose does not alter the inhibitory constant

of the free base (Table 8). However, addition of a phosphate group alters the inhibitory constants of these ligands, especially in the case of adenosine. The change of free energy in binding from inosine to IMP and from adenosine to AMP are 0.2 and 1.6 Kcal respectively. It is important to look into the reason for this significant difference between the energy requirement for the binding of the two molecules. Following the argument cited previously in this section, we can assume that 1) although in solution both inosine and adenosine favor an ANTI conformation, these nucleoside structures are rather free in converting from one conformation to the other, 2) addition of a phosphate group, however, fixes the nuclec-compounds in their preferred conformations which is ANTI for AMP and possibly SYN for IMP, 3) the binding site of these ligands favors stacking of SYN-conformers.

It can then be postulated that in order for AMP to be bound, it has to acquire a certain amount of energy to convert to a rather unstable SYN conformation, whereas in the case of IMP, this is unnecessary. The slight increase of the inhibitory constant of IMP with respect to inosine may be due to the electrostatic interaction between the phosphate and protein side chains (possibly glu287, glu381, glu571 and arg769, table6). the electrostatic interaction may be expressed by the equation(Schulz and Schirmer 1979)

TABLE 8
Kinetic data for the ligands studied
(Kasvinsky et al 1978b,c).

Ligands	Conc. mM.	Ki mM.	Delta Gd=RTLnKi
Caffeine	1.0	0.1	-5.5
FMN	1.0	0.01	-6.8
Hypoxanthine	0.9	1.1	-4.1
Inosine	1.0	1.1	-4.1
IMP	---	1.5	-3.9
Adenine	1.0	0.4	-4.7
Adenosine	1.0	0.4	-4.7
AMP	---	6.0	-3.0
ATP	---	60(10-20)	(-2.5)

$$E = \frac{332\, q_1 q_2}{\epsilon\, R_{12}}$$

Where q_i = partial charges in electron charges e .

R_{12} = distance between partial charges in Angstroms.

ϵ = dielectric constant

E = repulsion or attraction energy in Kcal per angstrom

if the dielectric constant is taken to be 30, the repulsion between two unit negative charges at distances of 3, 6, 9, 12 and 15 angstroms will be 3.7, 1.8, 1.2, 0.9 and 0.7 Kcal respectively. From table6 it is evident that there are four negatively charged amino acids (glu287, glu381, glu571 and asp768) and one positively charged amino acid (arg769) within 10 angstroms of O5' of inosine in the binding site, if a phosphate is attached to the O5', the net force can be

as high as 4 times 1.2 Kcal minus 0.9 Kcal, that is 3.9 Kcal, therefore the contribution of electrostatic forces to the binding of IMP would be significant. This value of 3.9 Kcal for a monopole approximation may account for the fact that ATP does not go into the binding site far enough and hence resulted in a weaker binding. However, due to the large number of charges involved, detailed calculation of the net force is difficult and has not been undertaken.

In the case of ATP, there is a variation in the reported Ki values between solution and crystal studies. Taking the results of crystal studies and assuming the mean to be 15 mM, we can show that the free energy required for the binding of ATP to the enzyme is 2.5 Kcal (Table 8). Therefore, difference in free energy of binding between ATP & adenosine is 2.1 Kcal , this may be the results of 1) electrostatic interaction, and/or 2) conformational transition. Further, the difference between binding of ATP and AMP indicates that the presence of two additinal phosphates does not affect the binding of the nucleotide to the enzyme tc any great extent once the molecule has been made "rigid" by the first phosphate.

Bio-energetics of ligand-Protein interaction

Since Kauzmann introduced the concept of hydrophobic bonding (Kauzmann 1959), it has been considered by some to be the most important force in protein folding, formation of protein multisubunit complexes, and binding of small

ligands. Using the concept of accessible surface area defined by Lee and Richards (1971) as illustrated in Fig.7, it was proposed that energy is released when protein accessible surface is "buried", that is, removed from contact with solvent; and the magnitude of this energy has been determined empirically as 25 cal per sq. angstrom of protein accessible surface area buried (Chothia 1974).

Janin and Chothia have recently applied this concept to study the binding of coenzymes to Flavodoxin (Janin and Chothia 1979), Lactate dehydrogenase and Glyceraldehyde-3-phosphate dehydrogenase. They calculated Delta G from the following equations:-

Delta G= Delta Grt + Delta Gh

Delta G = RT InKi

Where Delta Grt is the free energy loss in translational and rotational degrees of freedom, Delta Gh is the gain in entropy due to hydrophobic interaction and Ki is the inhibitory constant.

The first equation is derived from the classical thermodynamic equation Delta G = Delta H - T Delta S. The term T Delta S has been replaced by two components Delta Grt and Delta Gh. The first one gives a negative contribution while the latter gives a positive contribution to the entropy of the system. The enthalpy term Delta H in the equation has been assumed to be negligible, that is, no conformational changes or other energy interactions. For although hydrogen bonding and Van der Waals contact do exist

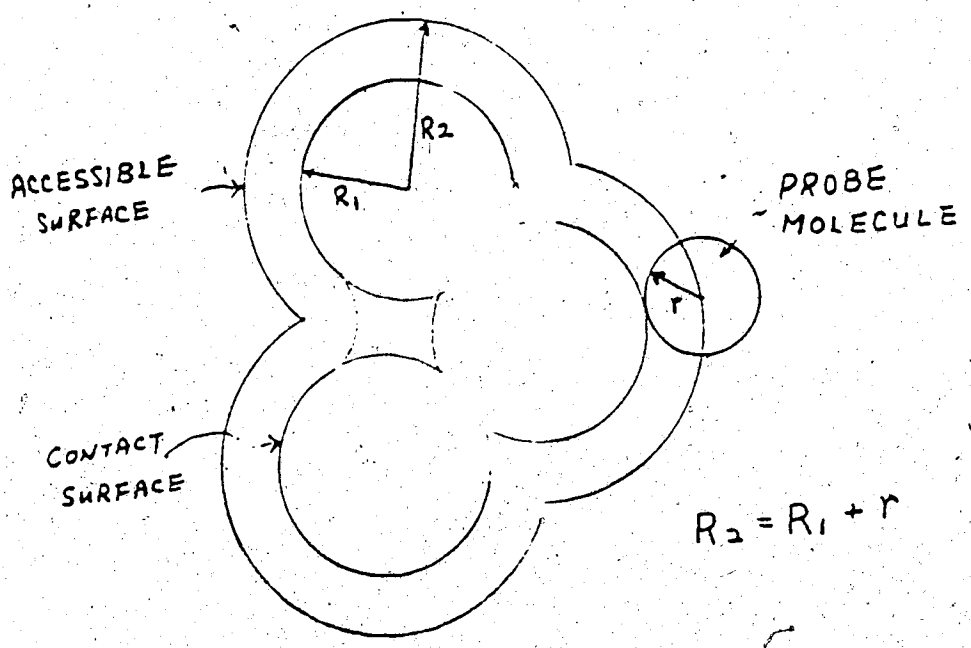


Fig. 7. This is to give an illustration of the accessible surface area as defined by Lee and Richards (1971)

between the protein and ligands, they would make similar interaction with the solvent when not bound, making that balance negligible. The value of Delta G as calculated from the two equations were comparable, hence justifying the omission of Delta H.

The same calculation has been carried out in our study using FMN, irosine and caffeine. Large discrepancies have been found in the Delta G calculated from both equations (T[illegible] The reason for the seemingly contradictary [illegible]een the two studies can be due to the following [illegible]e ligands studied by us were not buried as those in the previous study. Hence the hydrophobic interaction is not large enough to compensate

for the entrcpy loss of the co-enzymes involved. 2) Stacking plays an important role in the protein-ligand interaction energy in our study while this does not exist and so has not been considered in Janin's study. This stacking interaction is an enthalpy driven process and can not be accounted for by the hydrophobic energy alone. Therefore an appreciable Delta H term must be considered in the present study.

A simple rearrangement of the equations cited above would give the followings:-

$$-\Delta H = RT \ln K_i - T \Delta S$$

$$= RT \ln K_i - \Delta G_{rt} - \Delta G_h$$

If this Delta H is contributed solely by the stacking interaction, Delta H/2 would give us the stacking enthalpy, since the stacking interaction in our study involved 2 pair-stacking, which is tyrosine - ligand and ligand - phenylalanine.

The experimental and theoretical value of Delta H for stacking has been under extensive investigation by other workers (Ts'o et al 1963; Vorst et al 1965; Stoesser et al 1966; Gill et al 1967; Gupta et al 1978). No agreement has been arrived at due to the difficulties involved in its measurement. A number of values have been reported ranging from 3 Kcal/mole to 10 Kcal/mole. The Delta H/2 values obtained from our study have been listed in Table 7. They fall well within the reported range of values and agree quite closely with one another. This suggests that the stacking energy involved in the binding of these ligands

does not differ appreciably from one case to another. The difference of their binding constant is therefore contributed mainly by the difference in their entropy, which, in this case, is the rotational translational entropy and the hydrophobic entropy. Since Delta Gh represents the hydrophobic interaction between the ligand and the protein, the above observation suggests that the difference in the Ki of one system from another is determined mainly by the difference in the surface area of the ligand buried in the protein.

Mechanism of inhibition of enzymatic function.

From the above discussion it has gradually become apparent that stacking interaction plays an important role in selectivity of the nucleoside binding site. As mentioned in the introduction, the enzyme phosphorylase a exists in a state of T-R equilibrium in sclution. In a recent crystallcgraphic study with the enzyme, it was found that as the enzyme undergoes its T-R conversion induced by UDPG, one of its polypeptide loops which joins helix 265-275 and 290-310 is displaced from its "original" position as demonstrated in Fig 8. A close examination of a wire model suggests that as long as the "movable" loop remains in its "original" pcsition, the entrance of substrate into the active site would be blocked and hence the enzyme would be in its inactive form. In addition, the center of the pyrophosphate of UDPG is 2.0 angstroms from the OG of

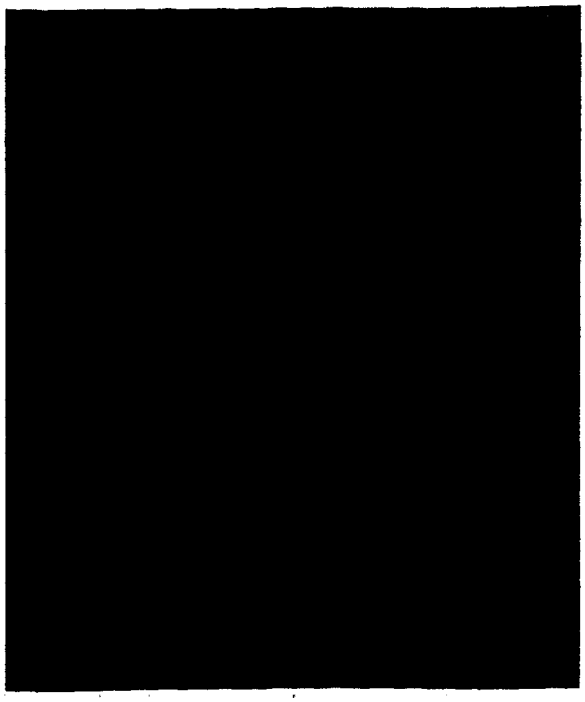


Fig. 8. Interpretation of the difference density map from UDPG experiment, red represents the negative contouring while blue represents the positive contouring. Usually when the chain movement is will defined, one can correlate the negative density with the positive density. However, in this case, the movable loop which is shown in dashes might likely becomes disordered so no positive density can be assigned to it as its final position.

Asp-283 of the (T) form which would suggest that the Asp-283 must move upon binding of UDPG, resulting in the movement of the whole loop.

One of the amino acids Phe-285 which is involved in the stacking complex is located in the movable loop. The presence of inhibitors attaches this Phe to Tyr-612 to form a stacking complex, thus fixing the "movable" loop to its "original" position. As a result, the T-form of the enzyme

is stabilized. The argument is supported by the finding that in Potato phosphorylase, which exhibits no regulation, the Phe-285 is replaced by Serine (Nakano et al 1978). However, this may not be the only mechanism contributing to this stability. It has been reported that this inhibition site is Synergistic with respect to glucose inhibition of phosphorylase a. In previous study, it has been observed that hydrogen bonding may exist between the C2 of the glucose and the ND of Asp-284, which is also located on the movable loop (Yang et al 1978 unpublished results). It can be postulated that this hydrogen bonding may give further stability to the loop and hence the enzyme in its T form. If this postulation is true, this can provide a possible explanation to the above mentioned Synergism.

It has been demonstrated that in liver phosphorylase a, a functional "nucleoside site" also exists, both in vitro and in vivo. This "nucleoside site" appears to have a higher selectivity than the muscle enzyme, for it only allows the binding of purine and purine analogues. However, it is questionable whether a physiological effector does exist which is able to bind to this "nucleoside site" in vivo (Kasvinsky et al 1978c). To answer this question, a number of experiments have been done in this laboratory by soaking crystals of muscle phosphorylase a in liver extract (Madsen and Fletterick unpublished). Subsequent crystallographic studies have revealed an unidentified ligand(s) which binds to this "nucleoside site". The electron density map, as

shown in Fig 9 suggests that the bound ligand is likely to be a purine analogue. Since the muscle enzyme used has a relaxed specificity on binding of effectors, it is possible that this density map may represent the average of a number of ligands of similar structures. Therefore any attempt to interpret this density map has to be done with caution. However, since the site may be involved in the insulin governed glucose homeostasis, it is expected that future identification of the effector in the physiological system will bring about significant insight on the understanding of this important hormonal regulatory system.

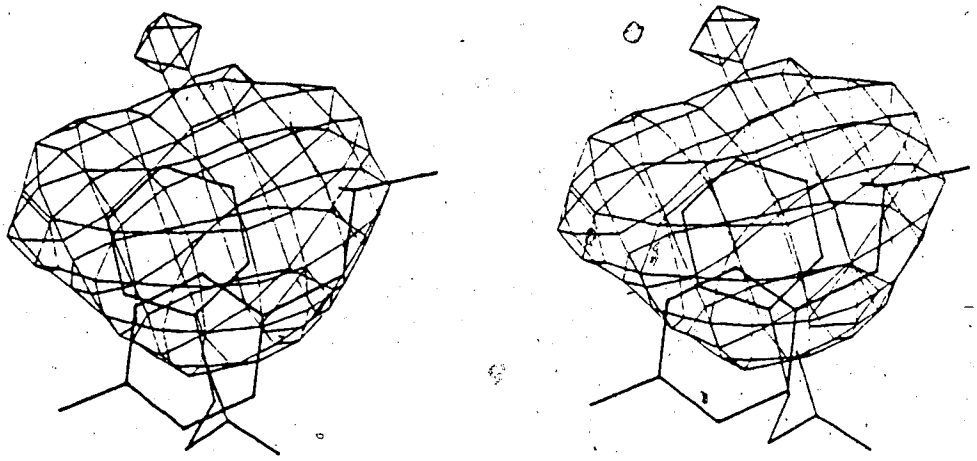


Fig. 9. Unknown ligands in the liver extract (see text).

D. Conclusion

In the present study, a number of fused-ring ligands have been used to investigate one of the regulatory sites of the muscle enzyme phosphorylase a. Although the study has been somewhat limited by the resolution of the difference electron density map due to the present MIR phases attainable, we were able to obtain a number of interesting findings on the energetics of the protein-ligands interaction as well as some aspects on the inhibition mechanism. However we have not been able to obtain a full understanding of the pattern involved in the stacking. This may be rendered possible in the future after the protein phases have been refined or with higher resolution Delta F maps of these and additional ligands.

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