Arch. Biol. Med. Exp. *19:* 77-83 (1986)
Printed in Chile

A model for the structure of fructose-1,6-bisphosphatase from pig kidney

Un modelo para la estructura de fructosa-1,6-bisfosfatasa de riñón de cerdo

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Fructose-1,6-bisphosphatase (EC. 3.1.3.11) is an allosteric enzyme that plays a key role in the gluconeogenesis process. It forms a tetramer of identical subunits, its action is inhibited by AMP, it requires the presence of a divalent cation to be active and it is proteolytically regulated. The prediction of the secondary structure of this enzime was done by the hydrophobicity profiles' method and by the Chou and Fasman's method with modifications. The predicted structure shows 38% β-structure, 22% helical structure and 4% β-turns. The structure can be described in terms of two domains joined by a 17-residues strand of random coiled structure. The location of the active and of the regulatory sites in the model proposed was made by secondary structure analogy with the enzyme obtained from rabbit liver. Domain I contains the AMP binding site and the proteolytic regulation site. Domain II has the active site, which, by appropriate superposition of both domains, can be located close to the AMP binding site and to the hiperreactive SH group. The model proposed meets several structural restrictions, it is thermodynamically stable and can explain the enzymatic behavior of the protein.

INTRODUCTION

Fructose-1,6-bisphosphatase catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate, reaction that is a key regulatory step in the gluconeogenesis process. The enzymatic reaction is inhibited by fructose 2,6-bisphosphate and allosterically inhibited by AMP. The enzyme has been described as a tetramer of identical subunits of molecular weight about 36000, presenting individual binding sites for the substrate and for the effectors.

Even though the enzyme, isolated from different gluconeogenic tissues, has been extensively studied (1, 2), the data about the active and regulatory sites amount only to the identification of some of the amino acid residues involved in them in the enzyme obtained from rabbit liver, and, by sequence homology, this identification has been extended to the pig kidney enzyme (3).

The complete primary structures are known for the sheep liver (4) and for the pig kidney enzymes (3), partial sequences have been determined on the enzymes obtained from rabbit, chicken, turkey and mice liver, and rabbit kidney (5). Even though crystals have been obtained from chicken (6) and rabbit liver (7) fructose-1,6-bisphosphatase, and recently from the pig kidney enzyme (8), no data about the tertiary structure is presently available.

The enzyme obtained from pig kidney has 335 amino acid residues in the subunit, with a molecular weight of 36534, calculated from the primary structure (3). It shows a great homology to the enzymes obtained from rabbit, sheep, mice or turkey gluconeogenic tissues (5).

The present paper reports the prediction of the secondary structure of the pig kidney enzyme by two prediction methods based on the amino acid sequence. A three dimensional model built according to the predicted secondary structure and to the chemical evidence available, allow us to propose reasonable locations for the active site, the site sensible to proteolysis, the allosteric site for AMP and a possible allosteric site for fructose-2,6-bisphosphate.

METHODS

Among the numerous methods that attempt the prediction of secondary structure of proteins from the amino acid sequence, there are several based in empirical or statistical probabilities, in numerical algorithms, or, in a combination of physicochemical measurements with a data base of known protein structures (9-12). Of them, two that have been reported to give 80% reliability when applied to globular proteins have been used in the prediction reported here. These were the hydrophobicity profiles' method (9) and the Chou and Fasman's method (10).

The hydrophobicity profiles' method gives the relative position of portions of the polypeptide chain with respect to the protein surface, since there exist a linear correlation between the "surrounding hydrophobicity" H_f –as defined in (13)– and the average distance of the amino acid residue to the protein surface, as measured on 21 known protein structures. The "surrounding hydrophobicity" for a given residue is defined as the sum of the Tanford hydrophobicities (13) of all the amino acids included in an 8 Å-radius sphere, centered at the alpha carbon of the residue in consideration. Since the calculation of the surrounding hydrophobicity implies the knowledge of the tertiary structure, a "bulk hydrophobic character" $<H_f>$ has been calculated as an experimental average value, from the tertiary structures available for 21 proteins (13). The hydrophobicity profile is simply a plot of $<H_f>$ versus the amino acid number in the sequence. Four basic profiles have been defined for 4 types of secondary structure: Helix, β-turns, buried and exposed β-strands (9); the identifications of these basic patterns in the hydrophobicity profile of the protein yields the predicted secondary structure.

Chou and Fasman's method to predict secondary structures defines conformational parameters P_α, P_β, and P_t for each of the 20 natural amino acids. These parameters represent the normalized frequence of occurrence of each amino acid residue in that particular type of secondary structure, as obtained from a data base of 29 fully determined protein structures. A probability average greater than 1.0, obtained for a group of amino acids taken in sequence (6 for a helix, 5 for a β-strand and 4 for a β-turn) is an indication that a certain type of structure is likely to occur in that region of the sequence. In order to improve the sensibility of the method in the vicinity of the limit value 1.0, the probability average can be replaced by a product of the conformational parameters (14). This and two other modifications of the method, one that consider 4 conformational parameters for each amino acid residue in a turn structure (15), and another that allows a diferentiation between β-strands participating in parallel or antiparallel β-structures (16) were used in the prediction reported here.

The prediction of the secondary structure was done independently by both methods, employing the primary structure of the enzyme reported by Marcus *et al.* (3). For a joint prediction, when discrepances were found between both methods, the results from the hydrophobicity profiles were preferred if the profile was a typical one. The discrepances amounted to no more than 19% of the amino acids.

A 3-dimensional model of the predicted secondary structure was built using rigid arrows, cylinders and "hairpins" to represent the β-strands, helical zones and β-turns respectively. These elements were joined by mobile connections and by flexible wire that represented the random coiled zones. The lengths of the building elements were scaled to the distances between the α carbons in that particular type of structure, and to the number of amino acids involved. The following complementary information was considered in building the model: distinction between exposed and buried β-strands (as predicted by the hydrophobicity profile); preferential participation of β-strands in parallel or antiparallel β-sheets, as given by Lifson and Sander (16); stabilization of β-strands and helices in one of the following super secondary structures: a $\beta\beta$ unit formed by two antiparallel β-strands, a $\beta\alpha\beta$ unit formed by a helix packed with two parallel β-strands,

and a $\alpha\alpha$ unit, which is made by two antiparallel helical structures (17).

Two "Greek key" models and the restrictions mentioned above gave rise to a 3-dimensional model for the subunit of fructose-1,6-bisphosphatase which presented two domains joined by a strand of random coiled structure.

The location of the active site and of the AMP site in this model was done by secondary structure homology with the enzyme obtained from rabbit liver, where the sequences in the neighborhood of these sites have been determined (18, 19). The region from the amino acid residue −79 to −39, in the rabbit liver enzyme, that has been postulated to be involved in the active site, presents a 75% homology with the sequence 249 to 290 of the pig kidney enzyme. Moreover, it shows almost 100% conservation of the secondary structure, since the amino acid replacement has been made by hydrophobically equivalent amino acid residues (Fig. 1).

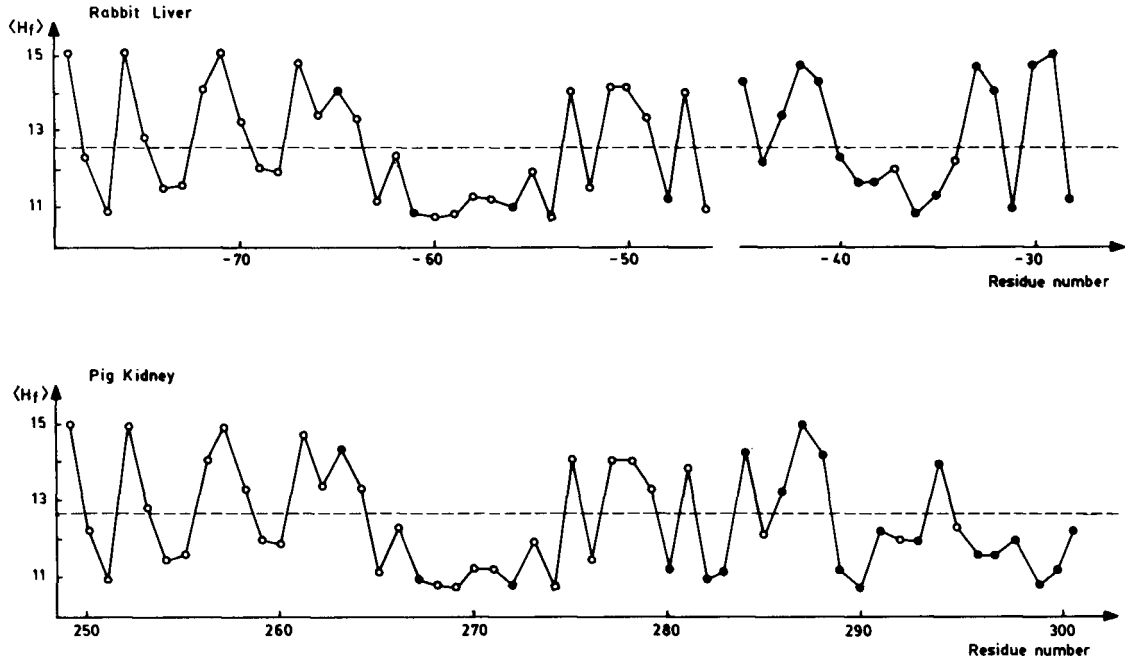


Fig. 1: Hydrophobicity profiles for the sequences −79 to −27 of the rabbit liver enzyme and 249 to 301 of the pig kidney enzyme. The bulk hydrophobic character $<H_f>$ is plotted against the amino acid number in the sequence. $<H_f>$ values for non-conserved amino acid residues are represented with filled circles. A deletion after residue −47 in the rabbit enzyme is postulated for better alignment. The important Lys −54 and its analog Lys 274, that should be part of the respective active sites, are marked with an open square. The limit between a hydrophobic and hydrophilic character was taken as 12.6 kcal which is the average value of the bulk hydrophobic character considering the complete sequence of the pig kidney enzyme. Note the identity of the profiles up to residues −39 and 290 respectively.

Pyridoxal phosphate modification of Lys 58 in the rabbit liver enzyme has shown that this residue is involved in the AMP binding site (19). A similarity between the sequences 51 to 83 of the rabbit enzyme with 135 to 166 of the pig kidney enzyme was established by a comparison of the hydrophobicity profiles rather than by a primary structure conservation: there is only 50% invariability in the sequences compared, however, the similarity between the profiles is evident (Fig. 2). Lys 141 would be the amino acid involved in the AMP binding for the pig kidney enzyme.

RESULTS AND DISCUSSION

The predicted secondary structure for the pig-kidney enzyme has a 38% β-structure, 22% helical structure and 4% of β-turns (Fig. 3). The enzyme subunit is organized

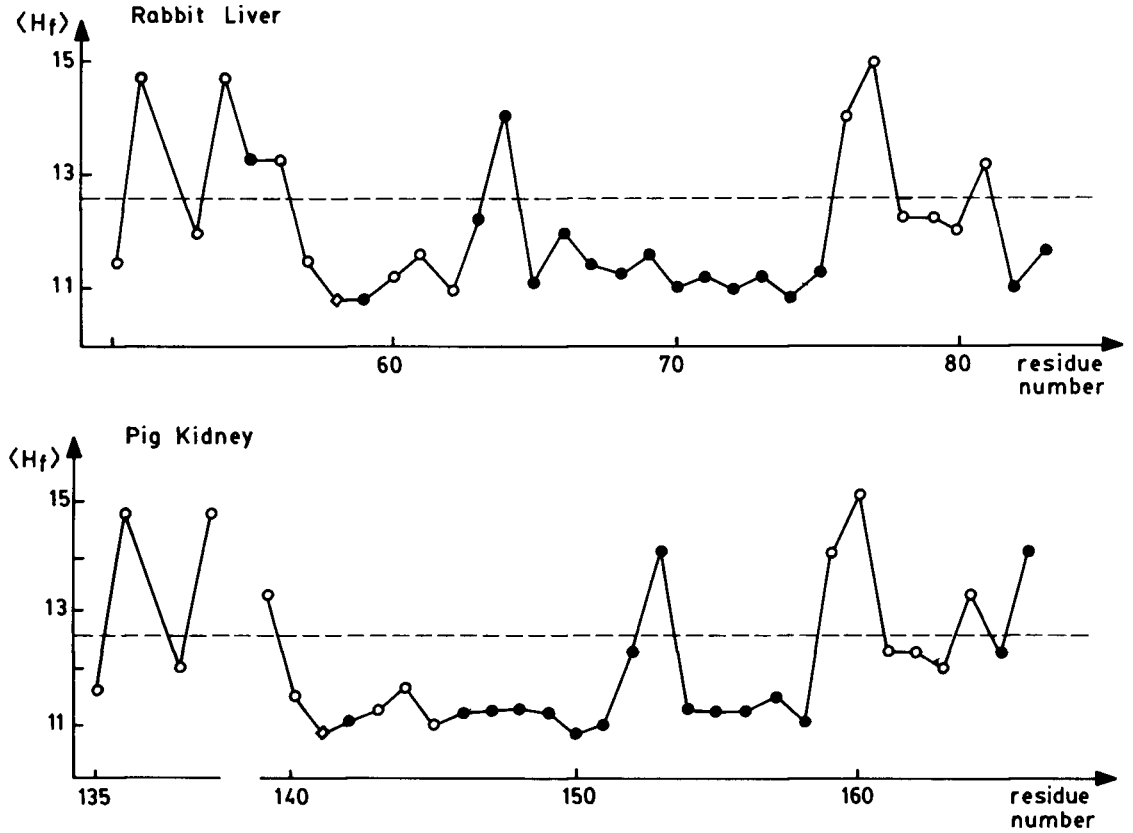
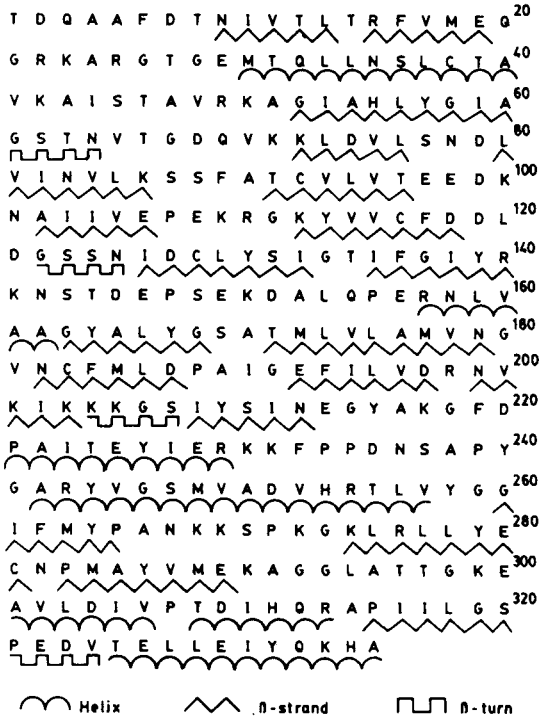


Fig. 2: Hydrophobicity profiles for sequences 51 to 83 of the rabbit liver enzyme and 135 to 166 of the pig kidney enzyme. Note the similarity between the profiles, even though the conservation of primary structure is low. Lys 58 and its analog Lys 141, marked with an open square are involved in the AMP binding site. The same nomenclature as in Fig. 1 has been used.



in two domains, as it would be expected for a globular protein of structural class α/β, with more than 300 amino acids (17). Both domains can be described as β-barrels formed by parallel and antiparallel β-strands (Fig. 4).

Domain I, formed by the first 141 residues, presents 10 β-strands, eight of them being stabilized by neighbouring antiparallel strands, and two parallel β-strands (β_2 and β_3) that are stabilized by the only helix of that domain (α_I). In this domain are located Lys 141, the binding site of AMP (analog to Lys 58 in the rabbit liver enzyme) (19); the zone sensible to proteo-

Fig. 3: Schematic diagram of the predicted secondary structure for the pig kidney fructose-1,6-bisphosphatase. The one letter symbol has been used in the primary structure representation according to the code: A, Ala; G, Gly; S, Ser; V, Val; L, Leu; I, Ile; P, Pro; F, Phe; W, Trp; M, Met; T, Thr; C, Cys; Y, Tyr; N, Asn; Q, Gln; D, Asp; E, Glu; K, Lys; R, Arg; H, His.

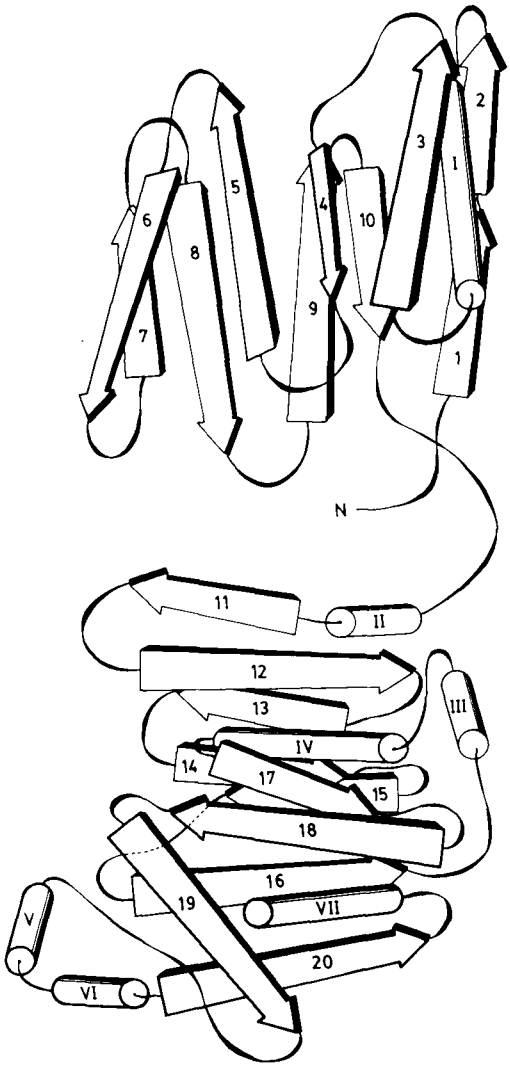


Fig. 4: Arrow and cylinder representation of the proposed two-domain structure for fructose-1,6-bisphosphate. The arrows represent the zones of extended structure (β-strands), and the cylinder, the helical zones. Note the β-barrel structure of each domain.

lysis, including residues 57 to 67 (20), and the hyperreactive SH group of Cys 128 (21).

Domain II includes amino acid residues 157 to 335, and presents 10 zones of extended structure and 6 helices. In this domain, 4 β-strands participate in folding units $\beta\alpha\beta$ (β_{12} α_{IV} β_{17} and β_{16} α_{VII} β_{20}) (17), and the rest in units $\beta\beta$ (stabilized by neighbouring antiparallel β-strands). The helical structures α_{II}, α_{III}, α_V and α_{VI} that do not participate in the stabilization of two parallel β-strands were located at the domain surface. In domain II is located Lys 274, which, being the analog of Lys −54 of the rabbit enzyme, should be a part of the active site (18).

Both domains are joined by a 17-residue strand of random coiled structure which allows the superposition of both domains in a way that permits the proximity of the AMP binding site, the active site and the hyperreactive SH group (Fig. 5). Also the zone sensible to proteolysis (residues 57 to 67), is exposed in the model proposed, as it has been stablished by several authors (20).

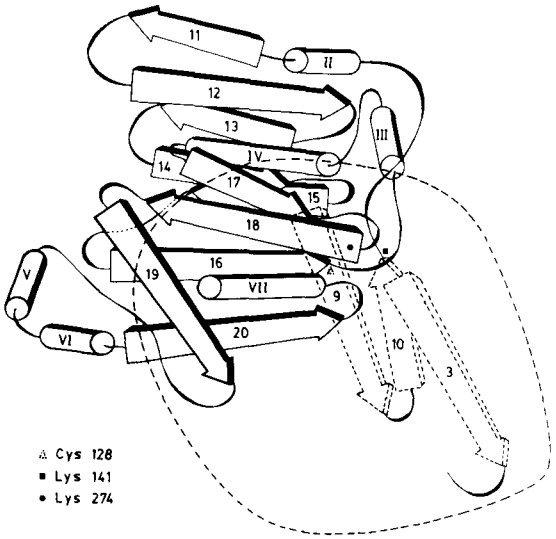


Fig. 5: Superposition of the two domains in the proposed model structure. Only the contour of domain I and three important β-strands, β_{10} preceeding Lys 141, β_9 containing Cys 128 and β_3 preceeding the loop sensible to proteolysis were represented, for the sake of simplicity.

The location of the active site and of the AMP side made by secondary structure homology, agree with that proposed by Marcus *et al.* (3), based on the primary structure homology found between pairs of hexapeptides in the pig kidney and rabbit liver enzymes.

The secondary structure of the sheep liver enzyme should be almost identical to that proposed here for the pig kidney protein, considering their similarity in primary structure (4). There are 31 changes, one deletion and two additions in the sequence of the sheep liver protein when compared to that of the pig enzyme; 26 changes are conservative, in the sense that

an amino acid residue has been replaced by another hydrophobically equivalent (9). The five non-conservative substitutions are Ala 5 by Pro, Asn 101 by His, Asn 142 by Ile, Ala 242 by Ser and Ala 334 by Thr. The secondary structure prediction, according to the hydrophobicity profiles, show only the following changes: β_7 would be one residue longer (due to the presence of His 101) and α_{VII} would be reduced to half of its length (due to the deletion of Tyr 331 and to the presence of Thr instead of Ala).

Fig. 6 illustrates the distances obtained in the proposed model between the AMP site, the active site and the hyperreactive SH group, which are in agreement with those obtained by ^{1}H-NMR and EPR studies on the divalent cation binding site on the rabbit liver enzyme (22). Also, ^{1}H-NMR and ^{31}P-NMR studies in the bovine liver fructose-1,6-bisphosphatase have given a similar distance between the active site and the hyperreactive SH group (23).

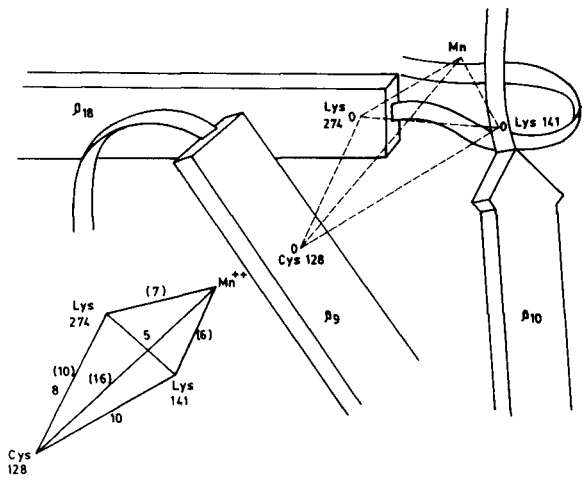


Fig. 6: Correlation between the distances measured in the proposed model and those determined by Cunningham *et al.* (22) by ^{1}H-NHR and EPR studies in the rabbit liver enzyme. Cunningham's values are represented between brackets. The distances are expressed in Å.

The inhibition mechanism of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate is controversial: some authors (24, 25) have reported the existence of an allosteric binding site, whereas others (26, 27), favour the interaction of this metabolite directly with the active site. A third position (28), postulates a biphasic behaviour of fructose-2,6-bisphosphate depending on the substrate concentration: low concentrations of fructose-1,6-bisphosphate would favour the interaction of fructose-2,6-bisphosphate with the active site, whereas at high concentrations of the substrate the metabolite would bind to its own allosteric site. These last results could explain the fact that NMR studies in the bovine liver enzyme suggest that fructose-2,6-bisphosphate is located at the active site, since they were made in the absence of the substrate (23).

A location for an allosteric site for fructose-2,6-bisphosphate can also be postulated in the model proposed here: this site should include the hyperreactive SH group of Cys 128, since modification experiments of this group both in the pig kidney (25) and in the rat liver enzymes (29) have shown that it is protected by fructose-2,6-bisphosphate. The distance between Cys 128 and the active site in the model would be 8 Å, thus, the proximity of both sites would give a reasonable explanation for the enzymes's inhibition by excess of substrate (30), assuming an interaction of fructose-1,6-bisphosphate with the allosteric site of fructose-2,6-bisphosphate. Also, the enzyme activation by this last metabolite at low substrate concentrations could be due to its interaction with the active site.

Recent X-ray diffraction studies of the pig kidney enzyme have shown the existence of three tetrameric units packed in a unit cell of space group P $3_1$21 (8). The unit cells contain twelve subunits and only six equivalent positions, thus, the asymmetric unit is formed by two subunits which may be related by a non-crystallographic 2-fold axis. The crystallographic 2-fold axis will complete a tetramer formed by subunits identical in pairs.

The dimensions of the subunit in the model proposed here are 45 x 40 x 26 Å. When the diffraction symmetry reported above (i.e. two perpendicular 2-fold axes) is applied to this model, a tetramer such as that shown in Fig. 7 can be obtained, with dimensions comparable to those reported for the pig, chicken and turkey enzymes

(6, 7, 8, 31). The location of the symmetry axes in Fig. 7 is arbitrary, we are aware that a better understanding of the rol that the tetramer fulfills in the enzymatic action or in the stability of the subunits is necessary to decide their precise location.

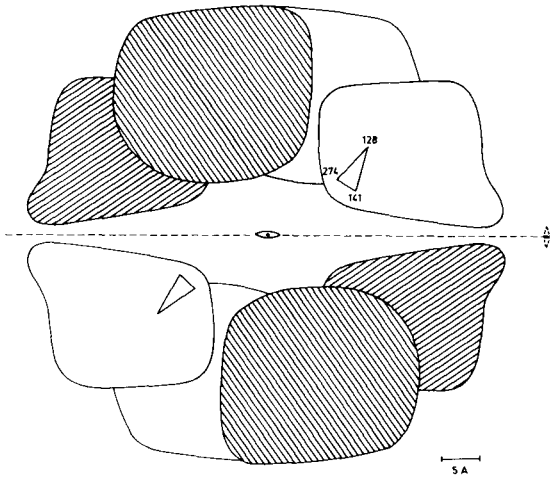


Fig. 7: A possible tetrameric structure for the pig kidney enzyme. This packing of the subunits will leave a free channel in the center of the tetramer. The location of the important triangle of Fig. 6 is marked. The position of the two perpendicular 2-fold axes that produced this tetramer is also shown.

In summary, a model for the structure of fructose-1,6-bisphosphatase that is thermodynamically stable is proposed. It fulfills several structural restrictions and can account for the enzymatic behaviour of the enzyme. Even though it is clear that the final word will be given by the complete determination of the tertiary structure by X-ray diffraction methods, this model gives new trends for the study of the regulatory sites of the enzyme, and, at the same time, it will facilitate the interpretation of the electron density maps when they will become available.

ACKNOWLEDGEMENTS

This work was partially supported by Research Project 20.33.16 from the Dirección de Investigación, Universidad de Concepción. Dra. Marta Bunster is gratefully acknowledged by a critical review of the manuscript.

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