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Studies with Type I Aldolase to Understand Fructose Intolerance and Combat Parasitic Disease

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Abstract

A structural study of the type I aldolases has been carried out to examine the isozyme specificity of these enzymes and the potential for designing specific inhibitors. Natural mutations in these aldolase enzymes are associated with haemolytic anaemia and fructose intolerance. It has also been proposed that inhibition of the parasitic version of the enzyme may provide a new lead in the design of drugs against malaria and sleeping sickness. X-ray crystallographic data is used with molecular modelling techniques to investigate the structural properties of these enzymes.

Aldolase is an enzyme of the glycolytic pathway, that catalyses the reversible reactions, production of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate from fructose-1,6-bisphosphate, production of dihydroxyacetone phosphate and glyceraldehyde from fructose-1-phosphate.

In man, the enzyme occurs in three different forms (isozymes). These have different substrate specificity and are located in specific regions of the body. The A isozyme is found in muscle tissue and erythrocytes. This shows a much higher catalytic activity for fructose 1,6-phosphate. The B enzyme is found in the liver and uses both substrates equally well. This is due to the enzyme being involved primarily in gluconeogenesis in the liver, whereas in muscle tissue and the erythrocyte it is involved in generating ATP via glycolysis. The final isozyme, the C isozyme is located in the brain.

The glycolytic function of these enzymes has been studied because of its importance in fructose intolerance (Cross et al 1988; Kaiser & Hegele 1991) and in haemolytic anaemia (Kishi et al 1987). Another interest in the aldolases is the potential development of a drug against protozoan infections such as malaria and sleeping sickness. The parasites that cause these diseases lack a citric acid cycle and so rely on glycolysis as their sole form of energy production. Inhibition or disturbance of the glycolytic pathway of these parasites would deprive the parasite of its energy source.

Studies of the human isozymes using X-ray crystallography and modelling techniques are being carried out to establish a structural link between the naturally-occurring mutations of the enzymes and the subsequent manifestation of the clinical conditions.

The X-ray structure of the human muscle isozyme has been solved to 2·2 Å (Gamblin et al 1990, 1991) and has provided the basis of the modelling studies. The sequences of the A and B isozymes have high sequence identity and so homology modelling was used to generate models of the B isozyme which will be verified by crystallographic data.

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Modelling studies are also being used to investigate the properties of the malarial enzyme with regard to the design of potential inhibitors. Primary sequence identity in this case is less than for the human liver enzyme but it is still above 60% and homology modelling has been used to generate a reliable model. One potential lead inhibitor is a nineteen residue peptide of the Band 3 membrane protein of human erythrocytes (Dobeli et al 1990). This protein is thought to be involved in the regulation of aldolase (Low 1986). It is significant that the peptide binds to the malarial aldolase with higher affinity than the human muscle aldolase, which is the normal form found in the erythrocyte. The peptide has been modelled to examine its conformational space.

Materials and Methods

Homology modelling of human aldolase B

Human aldolase B has 75% sequence identity with human aldolase A. The chains are of equal length and so there are no insertions or deletions. The B isozyme is therefore ideally suited for study using homology modelling (Greer 1991; Hilbert et al 1993). The primary sequences have been aligned for many Type I aldolase enzymes. These alignments have determined the nature of the sequence conservation by dividing it into regions which are totally conserved, conserved throughout one isozyme and variable regions (Kitajima et al 1990). This data is helpful in the assigning of structurally conserved regions.

With three crystal structures for Type I aldolases now known (Sygush et al 1987; Gamblin et al 1991; Hester et al 1991) it is possible to determine three-dimensional structurally-conserved regions by aligning the three dimensional structures. An alignment of two of the structures (those of *Drosophila melanogaster* and human muscle) within the molecular modelling package HOMOLOGY (version 2.3, Biosym Technologies, San Diego CA) yielded a very high degree of conservation throughout the protein. In the case of this study the differences are particularly important and so the structurally-conserved regions were assigned using the biochemical data. These biochemical studies (Kitajima

et al 1990) allowed the association of specific properties with the primary sequence. This was carried out by examining the properties of chimeric enzymes formed from combining lengths of the sequences of the A and B isozymes.

Coordinates were then assigned to those regions of the sequence which are structurally conserved by superimposition on a template crystal structure. In this case the human muscle protein was chosen as the template protein. This was carried out using HOMOLOGY. The resulting protein chain is incomplete and the non-conserved variable-loop regions were then built into the polypeptide chain using a database of the 60 best protein crystal structures to assign alpha carbon positions about which the amino acids can then be built. This process was accomplished in a single step within HOMOLOGY. Selection criteria for the generated loops depend on the root mean square deviation between the loop length and the distance between the broken ends of the chain. Loops were chosen from the database according to sequence lengths and similarity which is determined by root mean square deviation of model loop and database loop coordinates (the sequences alter and therefore alter the coordinates of the loop). It was more important to fit the gap distance than it is to obtain high sequence similarity in their coordinates. Strain can be introduced if this fitting of the loop is not reasonable.

Refinement of the model started with an examination of the splice points between the structurally-conserved regions and the built loop regions. This minimized the bond distance at these points to a peptide bond distance and constrained the peptide bond towards planarity. HOMOLOGY provides for the removal of bad intramolecular contacts using a database of side chains to minimize side-chain clashes. If clashes do exist, minimization can fail due to the massive energies produced by the van der Waals overlap terms. The contacts between the built loops and structurally-conserved regions were refined before the conformational flexibility of

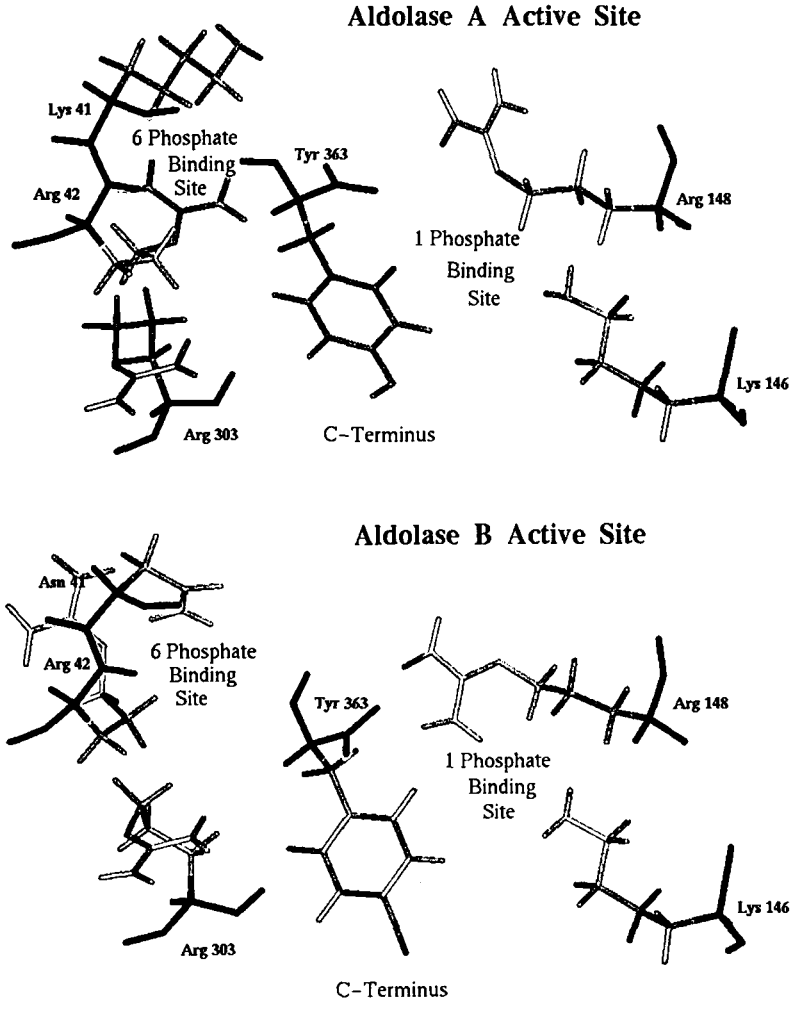

FIG. 1. A comparison of the active sites of the human A and B aldolase isozymes. The two diagrams illustrate the difference in structure of the active site between the human A and human B isozymes. The human B isozyme structure used here was produced by homology modelling. The active site is located towards the centre of the eight-stranded alpha-beta barrel structure where the C-terminus folds back into the barrel. It is a difference in the direction of approach of the C-terminus that is the main difference that can be seen at the top of the diagram. It is most probable that this is a result of the highly flexible nature of this region. The second region of obvious difference is to the left of the picture. This is the region of the 6-phosphate binding site. This is much more sterically hindered in the B isozyme. Graphical displays were printed out from the Insight II molecular modelling system.

the built loops was examined using the EXPLORE facility. The final model was then minimized until the maximum derivative of the energy function in x,y,z was less than 0·01 kcal mol^{-1} E.

Homology modelling of Plasmodium falciparum *aldolase*

Modelling of the malarial aldolase followed the same procedure as that for the human liver aldolase. The main exception was that the loop regions were also modelled within HOMOLOGY using an algorithmic loop generation procedure. This gives better fitting of the modelled loop to the gap but the loops produced in this way have a somewhat arbitrary conformation. This can be overcome at the later EXPLORE stage but it is a drawback of this method of loop generation. Another drawback is the size of the calculation for very long loops. Loop generation was used twice so that three models were produced for further refinement. One of the loop-generated models was used as a control for the refinement process and the only refinement used on it was the unrestrained minimization step. The other models were refined as before, starting with the repair of the splice points and finishing with the minimization step.

Molecular modelling of an inhibitory peptide

The inhibitor peptide (**B19**) was modelled using various conformation searching methods. These included the BOLTZMANN SEARCH procedure within the QUANTA (Molecular Simulations, Waltham MA) software package and various simulated annealing protocols within DISCOVER (Biosym Technologies, San Diego CA). The most complete exploration of conformational space proved to be a series of molecular dynamics simulations. Each of these was for 500 ps at 500 K. A high temperature was used to accelerate the simulation and to prevent the simulation becoming trapped in local minima. The results indicate some conserved structure even at such high temperatures. A group of solutions was then selected and taken through simulated annealing to give a final set of conformations which could be used for docking studies.

Results and Discussion

Homology models

To assess the quality of the homology models prior to the solution of the structure crystallographically is difficult. The models show good stereochemistry when assessed with the program PROCHECK (Laskowski et al 1993) but this in part results from the parameterization of the forcefields used to refine the model. The molecular energies of the model structures studied were all at about the same value of 1800 kcal mol^{-1} which shows that the models were consistent. This was also the energy found for the crystal structure of the human muscle enzyme after minimization with DISCOVER. The secondary structure of the muscle enzyme was unchanged by the minimization process. An assessment of the root mean square deviation between the model and the crystal coordinates indicated a close fit between the model proteins and the determined X-ray structure. Exceptions occurred at the protein, bulk solvent interface such as the flexible N-terminal region where deviations were high. These deviations are however to be expected. A final assessment of the success of this modelling strategy will only be possible when the determination of the X-ray structure of the liver aldolase enzyme has been completed. This determination is currently in progress and forms an integral part of the current study.

An examination of the active site in both the liver and muscle enzymes is presented in Fig. 1. This lends support to the belief that the model generated by this strategy is reliable and more importantly, useful. The residues are shown to be

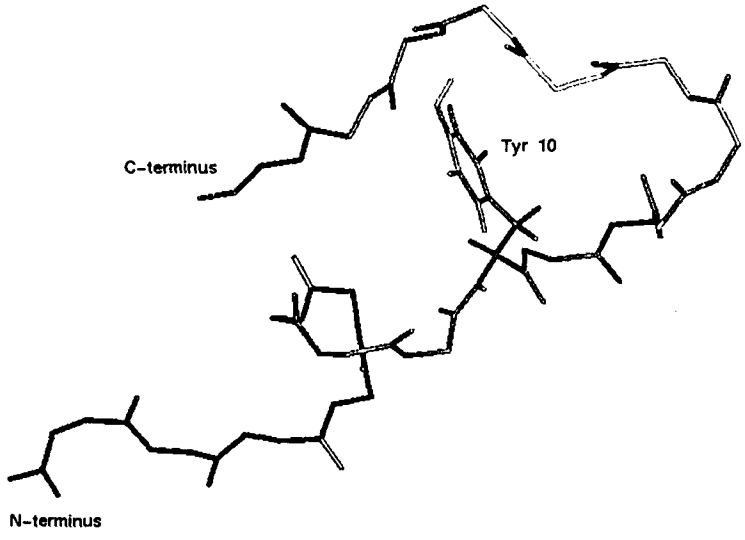

FIG. 2. A stable conformation of the 19-residue peptide inhibitor, showing the final conformation found for the peptide inhibitor after molecular dynamics simulation and simulated annealing. The starting point for this simulation was the linear peptide. This model exhibits significant secondary structure. There is evidence for an α-helix and a turn region. The tyrosine residue important for binding is to be found at the end of this helix as the turn region begins. This is particularly interesting as it suggests that the inhibitor requires the turn conformation which here is present in a linear peptide. It may therefore help in the design of further turn-mimicking peptide sequences.

well-conserved in their orientation, but the differences illustrated agree with the observations in kinetic experiments. Two main regions of difference shown in Fig. 1 are the C-terminus and 6-phosphate binding site. As well as steric factors, the substitution of a lysine for an asparagine at residue 41 will significantly alter the charge in this region making phosphate binding less favourable, the result of which will be no extra stabilization of the bisphosphate as compared with the monophosphate. This would be reflected by an equal activity for fructose 1-phosphate and fructose 1,6-phosphate as is indeed seen in the B isozyme.

Modelling of the inhibitor peptide

The conformational studies on **B19** have indicated that it may have a particularly stable conformational structure. This was anticipated by the fact that shorter inhibitors with the same recognition motif were not as effective. This suggested that either a large moiety was needed for inhibition or that the nineteen residue peptide exhibited some conformational structure not found in the shorter peptides. It is not unreasonable to assume that the longer peptide would retain a conformation closer to that of the corresponding region of the band 3 protein than the shorter peptide sequences would. It would therefore be a better mimic for the regulatory protein. Coupled with the increased possibility of intramolecular hydrogen bonding yielding elements of secondary structure, these results do not seem anomalous. One of the resulting final structures is illustrated in Fig. 2.

It is however important to run further simulations to verify that these results are not merely artifacts generated by the molecular mechanics forcefield. These artifacts can arise as a result of van der Waals collapse. This is when a particularly stable globular conformation is found for a peptide due to the over-weighting of the van der Waals terms of a molecular forcefield. Solvation has been one way to reduce this effect but this needs further verification as increasing the exchange of water molecules between solvating sites and bulk water will affect how the solvent modifies the van der Waals interaction. The effect of solvation has therefore yet to be determined.

Docking of such a large inhibitor is not possible without some knowledge of the protein inhibitor complex. The determination of the crystal structure is therefore necessary. This structure can then be used with molecular modelling procedures to investigate the nature of inhibitor protein interactions and, of particular importance, the conformation of the inhibitor. It is armed with such knowledge that further peptide inhibitors can be designed to maintain the conformation necessary for binding. This will allow the design of more potent and specific analogues which will provide a better lead for drug design.

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