Characterization of the allosteric binding pocket of human liver fructose-1,6-bisphosphatase by protein crystallography and inhibitor activity studies

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Abstract

The structures of three complexes of human fructose-1,6-bisphosphatase (FB) with the allosteric inhibitor AMP and two AMP analogues have been determined and all fully refined. The data used for structure determination were collected at cryogenic temperature (110 K), and with the use of synchrotron radiation. The structures reveal a common mode of binding for AMP and formycin monophosphate (FMP). 5-Amino-4-carboxamido-1 β -D-5-phosphate-ribofuranosyl-1H-imidazole (AICAR-P) shows an unexpected mode of binding to FB, different from that of the other two ligands. The imidazole ring of AICAR-P is rotated 180° compared to the AMP and FMP bases. This rotation results in a slightly different hydrogen bonding pattern and minor changes in the water structure in the binding pocket. Common features of binding are seen for the ribose and phosphate moieties of all three compounds. Although binding in a different mode, AICAR-P is still capable of making all the important interactions with the residues building the allosteric binding pocket. The IC₅₀ values of AMP, FMP, and AICAR-P were determined to be 1.7, 1.4, and 20.9 μ M, respectively. Thus, the approximately 10 times lower potency of AICAR-P is difficult to explain solely from the variations observed in the binding pocket.

Only one water molecule in the allosteric binding pocket was found to be conserved in all four subunits in all three structures. This water molecule coordinates to a phosphate oxygen atom and the N7 atom of the AMP molecule, and to similarly situated atoms in the FMP and AICAR-P complexes. This implies an important role of the conserved water molecule in binding of the ligand.

Keywords: AMP; AMP analogues; drug design; fructose-1,6-bisphosphatase; IC₅₀; X-ray crystallography

Noninsulin-dependent diabetes mellitus, also known as type II diabetes, results from a combination of insulin resistance at the target cells (liver, muscle, and fat) and relative deficiency in the insulin production from the β -cells of pancreas. The molecular

basis of NIDDM is poorly understood, but whatever the pathogenesis behind this serious condition is, it always manifests itself by hyperglycemia. Hyperglycemia is caused by reduced entry of glucose into various tissues and increased release of glucose into the circulation by hepatic gluconeogenesis (Consoli & Nurjhan, 1990). Therefore, reduction of the gluconeogenic rate in NIDDM may provide a novel treatment for NIDDM patients.

Fructose-1,6-bisphosphatase is the last specific enzyme in the gluconeogenic pathway, and thus plays a key role in the overall regulation of this pathway (Van Schaftigen et al., 1980; Pilkis et al., 1988). FB hydrolyzes fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. The enzyme is regulated (inhibited) by two endogenous ligands: fructose 2,6-bisphosphate and AMP. It is well established that fructose 2,6-bisphosphate is a competitive inhibitor of the substrate fructose 1,6-bisphosphate. The inhibition of FB activity by AMP is at the allosteric site and is synergistic with fructose-2,6-bisphosphate inhibition (Pilkis et al., 1988).

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Abbreviations: IC₅₀, inhibitor concentration required to elicit 50% inhibition of activity; FB, fructose-1,6-bisphosphatase; AMP, adenosine monophosphate; FMP, formycin monophosphate; AICAR-P, 5-amino-4-carboxamido-1 β -D-5-phosphate-ribofuranosyl-1H-imidazole; PEG, polyethylene glycol; DTT, dithiothreitol; FB:AMP, fructose-1,6-bisphosphatase and adenosine monophosphate complex; FB:FMP, fructose-1,6-bisphosphatase and formycin monophosphate complex; FB:AICAR-P, fructose-1,6-bisphosphatase and 5-amino-4-carboxamido-1 β -D-5-phosphate-ribofuranosyl-1H-imidazole complex; NIDDM, noninsulin-dependent diabetes mellitus.

The amino acid sequence of the human liver FB was determined recently and was found to be approximately 90% identical to the porcine kidney enzyme (Elmaghrabi et al., 1993). The human FB enzyme is a tetramer consisting of four identical polypeptide chains of 337 amino acids. The pig kidney FB has been studied extensively previously. X-ray structures of porcine FB complexed with the substrate product fructose 6-phosphate (Ke et al., 1991b) and the substrate analogues 2,5-anhydro-D-glucitol-1,6-bisphosphate (Villeret et al., 1995) and fructose 2,6-bisphosphate (Liang et al., 1992) have been reported. The catalytic mechanism and binding pocket have been well described, including the nature and position of the two divalent cations (Mg^{2+} or Zn^{2+}) required for catalytic activity in the porcine enzyme (Zhang et al., 1993; Villeret et al., 1995). The number and nature of the divalent cations required in the human liver enzyme have not yet been fully investigated. One and two Mg^{2+} ions have been found in the human enzyme (Gidh-jain et al., 1994), and similar observations have also been reported for the porcine enzyme (Zhang et al., 1993). The allosteric mechanism has also been investigated. The tetramer can be divided into two dimers, an upper dimer (A and B subunit) and a lower dimer (C and D). The subunit labeling (A, B, C, and D; also known as C1, C2, C3, and C4) follows the nomenclature given by Liang et al. (1993). A 16–17° rotation of the lower dimer (subunit C and D) relative to the upper dimer (subunit A and B) has been described for the porcine FB upon binding of AMP to the allosteric binding pocket (Ke et al., 1991a). The unrotated form is called the R form and the rotated form is called the T form. The only structures reported of FB complexed with an allosteric inhibitor is with AMP [porcine FB (Ke et al., 1991a) and human FB enzyme (Gidh-jain et al., 1994)]. Thus, more knowledge is needed about the allosteric binding pocket of human FB to fully understand the nature and function of the allosteric inhibition. The inhibitors used in this study are AMP and AMP analogues. Because the coordinates of the reported human FB:AMP complex (Gidhjain et al., 1994) were not available at the time of this study, the crystal structure of this complex was solved independently and it is included in this study to compare the structures and binding characteristics of the different inhibitors based on equally treated data sets.

Results

The overall structure of the human FB enzyme (Fig. 1) is similar to that of porcine FB (Ke et al., 1991a). No major changes are observed in the overall structures for the present three different complexes. In Ramachandran plots, made by the use of PROCHECK (Laskowski et al., 1993), all torsion angles fall within the allowed regions for all three structures (data not shown). Crystallographically, the four subunits of the tetramer are not identical, and hence slightly different numbers of residues were built in each subunit for each structure (Table 1). Electron density is lacking for all of the structures around residues 1–9, 62–71, 123–128, and 336–337. Map averaging (Kleywegt & Jones, 1993) did not improve the electron density in these areas. These flexible domains are spotted easily in the real space fit plots [made by the use of O (Jones et al., 1991)] (results not shown). Also, the loops around residues 268–271 are poorly defined in all three structures. Similar regions of flexible or disordered loops are reported for the porcine FB (Ke et al., 1990). The real space fit plots show that the higher-resolution FB:FMP structure is by far the best, and the correlation is very much dependent on the resolution, as expected. The average *B* values for each of the subunits are increasing from subunit A to D

Fig. 1. Structure of the human FB:AICAR-P complex. The tetramer consists of four identical polypeptide chains (A, B, C, and D), each of 337 amino acids. The AMP analogue AICAR-P (in red) bound to the allosteric binding pocket. Loops, white; α -helices, blue; β -sheets, yellow.

for the AMP and FMP complexes (Table 1). An overall *B*-factor was calculated for the FB:AICAR-P complex. Attempts to refine individual atomic *B*-factors using PROLSQ from the CCP4 suite of programs (CCP4, 1994) resulted in unrealistically low *B* values for some atoms in the AICAR-P complex. Still, the overall *B*-factor (16 Å²) for the AICAR-P complex is relatively low. There is a correlation between the main-chain *B*-factors and the RS fit (results not shown). The flexible loops with poor real space correlation also show high *B*-factors. In addition, the N and C termini have high *B*-factors.

Similar to the porcine FB structure (Ke et al., 1991a), divalent cation binding sites are found in the present structures. For the

Table 1. Residue number and *B* average per subunit

Complex	Subunit A	Subunit B	Subunit C	Subunit D
Number of residues built per subunit				
AMP	320	314	312	311
AICAR-P	320	312	311	312
FMP	320	316	312	311
<i>B</i> average per subunit				
AMP	21	23	26	31
FMP	15	16	18	21
AMP (pig) ^a	16	15	23	24

^aKe et al., 1991a.

FB:AMP complex, six Mg^{2+} ions are found. The A and B subunit each have one Mg^{2+} ion, located in the active site, the same site as the high-affinity site in the porcine FB structure (Zhang et al., 1993). In the C and D subunits, two Mg^{2+} ions are found in each subunit, one in the same high-affinity site as in the A and B subunits, and one in the low-affinity site, as described in the porcine FB structure (Ke et al., 1991a). The occupancy and *B*-factors of the six ions were estimated using MLPHARE (from CCP4, 1994). All ions, except the two Mg^{2+} ions in the low-affinity site, have an occupancy of approximately 1 (1.03, 1.10, 1.05, and 1.11 for the A, B, C, and D subunits, respectively). The occupancy in the low-affinity sites of the C and D subunits were 0.93 and 0.85, respectively. In the FB:FMP and FB:AICAR-P structures, only two Mg^{2+} ions were found, both situated in the A and B subunits at the high-affinity site, with an occupancy of approximately 1 (1.03 and 0.99 for the A and B subunits, respectively, in the FB:FMP structure and 1.01 and 1.05 for the A and B subunits, respectively, in the FB:AICAR-P structure).

The quaternary structural change during the R to T transition, involving a rotation of the lower dimer (subunit C and D) relative

to the upper dimer (subunit A and B) was examined using LSQKAB (from CCP4, 1994). Because the structure of an R form of the human FB has not been determined, an exact rotation value cannot be measured. Fitting first the two upper dimers of two structures, followed by a second fitting of the two lower dimers, one can measure if two different complexes have the same R to T transition rotation. All three structures have the same rotation.

The structures of the three inhibitor complexes have been refined at varying resolutions (Table 2). However, in each case, the positions, orientations, and conformations of the inhibitors within the binding pocket have been determined unambiguously. The quality of the information may be judged by difference Fourier electron density maps for which the inhibitors have been omitted from the phasing models (Fig. 2). After removing the inhibitor from the phasing model, several cycles of refinements were performed.

In all three inhibitor complexes analyzed, the inhibitors are bound to the allosteric site, situated some 28 Å from the active site. The binding involves residues Val 17, Thr 27, Gly 28, Gly 29, Leu 30, Thr 31, Lys 112, Tyr 113, Arg 140, Val 160, and Met 177. For detailed information, see Table 3 and Figure 3. To examine whether

Table 2. Crystal data and statistics for data collection and refinement

	FB:AMP	FB:FMP	FB:AICAR-P	FB:AMP
Data collection details				
Data collection site	EMBL Hamburg	EMBL Hamburg	EMBL Hamburg	York
Wavelength (Å)	0.927	0.927	0.927	1.57
Collimation (mm)	0.3	0.3	0.3	0.32
Number of crystals	1	1	1	1
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell (<i>a</i> , <i>b</i> , <i>c</i> in Å)	66.7, 82.6, 273.6	66.9, 82.9, 274.9	67.2, 83.3, 276.9	68.3, 86.8, 280.4
Resolution range (Å)	20.0–2.3	20.0–2.0	20.0–2.6	20.0–2.7
Unique reflections	66,503	104,969	45,532	41,814
Completeness	97.4%	96.3%	93.3%	92.1%
Reflections with $\langle I/\sigma(I) \rangle > 3$	70.0%	77.1%	69.4%	69.7%
R_{merge} (%) ^a	9.2%	7.3%	12.1%	9.1%
Outer resolution shell				
Resolution range (Å)	2.4–2.3	2.1–2.0	2.7–2.6	2.8–2.7
Unique reflections	7,433	18,532	4,135	5,103
Completeness (%)	92.1%	96.1%	77.5%	79.0%
Reflections with $\langle I/\sigma(I) \rangle > 3$	41.1%	52.9%	41.8%	39.3%
Refinement statistics				
Resolution range (Å)	10.0–2.3	10.0–2.0	10.0–2.6	10.0–2.7
<i>R</i> -factor ^b	19.0%	18.9%	18.6%	20.2%
Protein atoms	9,649	9,664	9,631	9,696
Inhibitor atoms	96	96	92	96
Water molecules	575	799	558	272
RMS bond length deviation (Å)	0.018	0.017	0.020	0.022
RMS bond angle deviation (°)	2.8	2.4	2.9	2.8
Mean <i>B</i> -factor (Å ²) ^c	25/29	17/22	16	27/33
RMS <i>B</i> -factor deviation (Å ²) ^d	1.3/2.9	1.2/2.7		1.2/3.4

$$^a R_{\text{merge}} = \frac{\sum |I - \langle I \rangle|}{\sum \langle I \rangle}$$

$$^b R\text{-factor} = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}$$

^c Average *B* value for main-chain and side-chain atoms, respectively. For the AICAR-P structure an overall *B*-factor was calculated.

^d Average RMS *B*-factor values for main-chain and side-chain atoms, respectively.

the three inhibitors bind to the enzyme in similar modes, considering hydrogen bond distances, the structures were superimposed (Fig. 4). The AMP, AICAR-P, and FMP molecules occupy the same space in the binding pocket. The phosphate and ribose groups are positioned almost identically in all structures. However, the conformation of the base of AICAR-P (the imidazole ring) differs from the conformations of the AMP and FMP bases. The imidazole ring of AICAR-P should be rotated approximately 180° (torsion 1) and, subsequently, the carboxamido group another 180° (torsion 2), to have the same base orientation as AMP and FMP (Fig. 5). The ambiguity in determining the orientation of the 4-carboxyamido group of AICAR-P based on the electron density can be resolved by looking at the hydrogen donor/acceptor capabilities in this particular area.

The water molecule coordinating to the phosphate oxygen atom and the N7 atom of the AMP structure is conserved in all three structures. Furthermore, this water molecule (referred to as the central water molecule) is the only water in the allosteric binding pocket that is conserved among all four subunits in all three structures. This implies an important role in ligand binding.

Although binding differently, AICAR-P is still capable of forming a similar hydrogen bonding pattern and van der Waals' contacts as AMP and FMP. Minor changes are seen around the central water molecule (Fig. 6). The hydrogen bond between this water molecule and the hydroxy group of Thr 31 is lost, but one is gained to Thr 31 N, keeping the total number of possible hydrogen bonds at four, the same number seen for the FB:AMP structure. The FB:FMP central water molecule structure differs from the FB:AMP structure. A total of six possible hydrogen bonds are found. The two additional bonds are seen to N8 of FMP and N of Thr 31.

The crystal structure of the inhibitor AICAR-P is known (Admick & Saenger, 1979) and is deposited in the Cambridge Structural Database (CSD) (Allen & Kennard, 1993) as entry ARFIMP10. Superimposition of the atoms of the ribose ring of AICAR-P (CSD) to the riboses of the ligands in the FB complexes reveals that the CSD structure resembles the AMP and FMP structures closely (Fig. 7). The AICAR-P (CSD), AMP, and FMP bases are oriented in the same way. A conformational grid search has been performed with SYBYL (Tripos Associates, Inc.) for all of the ligands. Torsion 1 (Fig. 5) of AMP and FMP, and torsion 1 and 2 of AICAR-P were rotated in steps of 15° . PM3 semi-empirical charges were used (Stewart, 1990). Two energy minima are present for the AMP and FMP structures and four for AICAR-P (data not shown). The AMP and FMP structures are binding to FB in a conformation close to the global energy minimum conformation of these structures (torsion 1 approximately 0°). In contrast, the binding conformation of AICAR-P (torsions 1 and 2 being -160° and 140° , respectively) corresponds to a local energy minimum, being a few kcal/mol higher in energy than the global energy minimum. The global minimum of AICAR-P (torsions 1 and 2, 0° and 0° , respectively) does, in fact, correspond quite closely to the AICAR-P (CSD) structure.

Fig. 2. $|F_o| - |F_c|$ difference Fourier maps (omit maps) for the three structures, contoured at 3σ level. In each case, the inhibitor has been omitted from the phasing models. After removing the inhibitor from the phasing model, several cycles of refinements were performed. Atoms are colored according to atom type (carbon and phosphorus, yellow; oxygen, red; nitrogen, blue).

Table 3. Hydrogen bond lengths for the inhibitor/protein contacts

Bond	Subunit A	Subunit B	Subunit C	Subunit D	Average ^a
FB:AMP					
N7-H ₂ O	2.5	2.5	2.6	2.5	2.5
N6-O Val 17	2.6	3.3	2.9	2.9	2.9
N6-Oγ1 Thr 31	2.6	2.8	2.6	2.6	2.7
O2'-H ₂ O	—	—	—	3.1	—
O3'-OH Tyr 113	2.8	3.0	2.6	2.6	2.7
O3'-NH ₂ Arg 140	—	—	2.7	2.9	—
O1A-Nz Lys 112	3.3	3.1	3.1	3.0	3.1
O1A-OH Tyr 113	2.4	2.5	2.6	2.5	2.5
O2A-H ₂ O	2.7	3.0	3.2	—	—
O2A-Oγ1 Thr 27	3.1	—	—	—	—
O2A-N Thr 27	2.9	2.7	2.9	3.0	2.9
O2A-Nz Lys 112	—	—	2.9	—	—
O3A-H ₂ O	2.4	2.5	2.5	2.6	2.5
O3A-N Gly 28	3.2	—	—	—	—
O3A-N Gly 29	2.9	3.2	3.3	3.0	3.1
O3A-N Leu 30	3.1	2.9	3.1	2.9	3.0
FB:FMP					
N1-H ₂ O	2.7	—	—	—	—
N8-H ₂ O	3.2	3.3	3.1	3.2	3.2
N3-H ₂ O	2.6	2.9	2.5	2.9	2.7
N7-H ₂ O	2.6	2.6	2.5	2.6	2.6
N6-O Val 17	2.7	2.9	2.9	2.8	2.8
N6-Oγ1 Thr 31	2.7	2.7	2.6	2.8	2.7
O3'-H ₂ O	—	—	—	2.8	—
O3'-OH Tyr 113	2.6	2.6	2.6	2.5	2.6
O3'-NH ₂ Arg 140	2.9	2.5	—	—	—
O2'-H ₂ O	2.7	—	—	—	—
O2'-NH ₂ Arg 140	3.2	—	—	—	—
O1A-Nz Lys 112	2.6	2.5	2.6	2.6	2.6
O1A-OH Tyr 113	2.6	2.6	2.5	2.5	2.5
O2A-H ₂ O	—	2.7	2.8	2.7	—
O2A-N Thr 27	2.6	2.6	2.7	2.7	2.6
O2A-Oγ1 Thr 27	2.9	2.9	2.6	2.8	2.8
O3A-H ₂ O	2.7	2.6	2.5	2.6	2.6
O3A-N Gly 29	2.6	3.1	3.0	2.8	2.9
O3A-N Leu 30	2.6	2.7	2.8	2.8	2.7
FB:AICAR-P					
N3-H ₂ O	2.6	2.5	2.5	2.5	2.5
N4-O Val 17	2.7	3.5	3.5	2.9	3.1
N4-N Glu 21	—	3.0	—	—	—
N4-Oγ1 Thr 31	3.0	3.1	3.0	2.7	3.0
O5-H ₂ O	2.5	2.6	—	2.6	—
O3'-OH Tyr 113	3.0	2.6	2.5	2.6	2.7
O3'-NH ₂ Arg 140	2.7	—	—	2.8	—
O2'-H ₂ O	3.2	—	2.6	2.6	—
O2'-NH ₂ Arg 140	3.1	—	—	—	—
O1A-Nz Lys 112	3.2	2.8	3.5	3.1	3.2
O1A-OH Tyr 113	2.5	2.6	2.6	2.2	2.5
O2A-H ₂ O	2.6	2.7	3.0	2.7	2.8
O2A-N Thr 27	2.8	2.8	2.6	2.7	2.7
O2A-Oγ1 Thr 27	—	—	2.8	—	—
O3A-H ₂ O	2.9	2.5	2.6	2.7	2.7
O3A-N Gly 28	—	3.2	—	—	—
O3A-N Gly 29	3.1	3.1	3.1	2.8	3.1
O3A-N Leu 30	3.1	2.9	3.1	2.9	3.0

^aAn average value is only given if the hydrogen bond is present in all four subunits. Distances beyond 3.5 Å were not considered as hydrogen bonds and are not included.

The IC₅₀ values for several inhibitors were measured by FB enzyme activity (Table 4). Removal of the 3-hydroxyl group of AMP does not seem to have any influence on the inhibitor activity. In fact, 3-deoxy-AMP is a slightly more potent inhibitor than AMP. Also, FMP is a more potent inhibitor than AMP. The inhibitory effect of AICAR-P is 12 times lower than of AMP. Substitution of the N7 of AMP with a carbon atom (7-deaza-AMP) results in no inhibitory effect observed (IC₅₀ > 500 μM).

Discussion

The inhibitor assay showed that AICAR-P is about a 10 times less potent inhibitor than AMP and FMP (Table 4). Dealing with an allosteric enzyme, the affinity of the inhibitors cannot be derived from the IC₅₀ value. In principle, all of the inhibitors might have the same affinity for the allosteric binding pocket. As described, AICAR-P's binding to FB differs from that of AMP and FMP in the orientation of its base. However, AICAR-P still makes all the important hydrogen bond contacts, indicating that the affinity of AICAR-P might be in close range to the affinity of AMP and FMP.

The inhibition of FB can, at least conceptually, be divided into two separate steps: a binding of the ligand in the binding pocket, and, secondly, an activation of the allosteric mechanism, leading to the inhibition of the enzyme. In the case of porcine FB, the allosteric mechanism involves an R to T transition (Zhang et al., 1994). The quaternary structural change during the transition involves a relative 16–17° rotation of the lower dimer (subunit C and D) with respect to the upper dimer (subunit A and B). This rotation was determined to be similar for all three inhibitor complexes of human FB. Thus, AICAR-P appears to invoke the same degree of activation of the allosteric mechanism.

The conformational analysis of the inhibitors reveals that, whereas AMP and FMP bind to FB in their global energy minimum conformations, AICAR-P binds in a local energy minimum conformation. The lower population of the binding conformation of AICAR-P might account for some of the reduced inhibitory activity.

The hydrophilicity of the three inhibitors used in this study might be another factor influencing binding. Going from the soluble state in the aqueous solution to the bound state in the protein, water molecules must be displaced. Looking at the three inhibitor structures, it appears that AICAR-P is the most hydrophilic ligand of the three, hence reducing its affinity to the binding pocket.

Only one water molecule (the central water molecule) was found to be conserved in the allosteric binding pocket. This water molecule coordinates to the phosphate oxygen atom and the N7 atom of the AMP molecule, and to similar atoms in the FMP and AICAR-P molecules (Fig. 6). The central water molecule has the possibility of making an extra hydrogen bond to the N8 atom of the FMP ligand in the FB:FMP complex. In all three complexes, the central water is within hydrogen bond distance of N of Gly 28 and, in the FB:AMP and FB:FMP structures, it is furthermore within reach of Oγ1 of Thr 31. Finally, it is within hydrogen bond distance from N of Thr 31 in the FB:FMP and FB:AICAR-P structures. In total, the central waters of the FB:AMP, FB:FMP, and FB:AICAR-P structures are involved in 4, 6, and 4 possible hydrogen bonds, respectively. The FB:FMP and FB:AMP central water is capable of making the same hydrogen bond patterns, whereas this is not the case for the FB:AICAR-P central water (Fig. 3). The compound 7-deaza-AMP is incapable of forming the N7 hydrogen bonding to the central water molecule (replacement of N7 with a carbon atom). This compound failed to show any inhibitory activity. Inhibition

Fig. 3. Schematic drawings showing the inhibitors and their interactions with the FB enzyme. Only the hydrogen bonds observed in at least three of four subunits are shown. Average hydrogen bond lengths are given in the drawing.

Fig. 4. Two different views of the allosteric site after superimposing the three inhibitor complexes.

might be sensitive to changes around the central water, indicating a possible role in binding of the ligand. For future inhibitor development, it might be advantageous to incorporate the central water molecule into the inhibitor structure.

Two metal binding sites in the active site of porcine FB have been reported (Zhang et al., 1993). In the present structures, either one or two magnesium ions are found in the different subunits. The asymmetric binding of Mg^{2+} is seen only across the A/B-dimer C/D-dimer interface. Asymmetric binding of Mg^{2+} has been reported for the porcine FB structure (Ke et al., 1991b), but between the A and B subunits and not between the A/B and C/D dimer, which are related by crystallographic symmetry in most of the work done previously with the porcine FB. The dimer-dimer asymmetry might only be found in the human FB, explaining why the human enzyme, so far, has not been crystallized in a space group with only one dimer per asymmetric unit.

The observed increase in the average *B*-factor per subunit (increasing from subunit A to subunit D) observed for the FB:AMP and FB:FMP is a finding that might be caused by negative cooperative binding. The observation cannot be explained or biased by the structure solution, because a monomer was used as a search model for the molecular replacement solution, and all the *B*-factors were set to 15 Å² prior to refinement of the atomic *B*-factors. It is known that crystal packing effects can influence the stability of different subunits or regions of a protein. However, the same pat-

tern of *B*-factor variation was observed in the porcine FB structure (Ke et al., 1991a), which crystallizes in a different unit cell. Also, examining the crystal packing, no subunits seem to be favored by having more crystal lattice contacts. In a large number of structures, it has been observed that ligand binding has a stabilizing effect on protein structure (Creighton, 1984). If the inhibitors are bound to the subunits in sequence from A to D, or first to subunits A and B and secondly to subunits C and D, the binding pockets of subunit A and B might have a higher degree of occupancy and hence higher stability than subunit C and D. In fact, the $2|F_o| - |F_c|$ electron density maps, contoured at 3σ level or higher, show a much more continuous density for the inhibitors in subunit A and B than in subunit C and D for all three structures (results not shown). Furthermore, it was possible to build more residues into subunit A and B than C and D (Table 1), consistent with the stability increasing from subunit A to D.

Our results are reasonably consistent with previous findings with respect to the AMP binding pocket (Gidhjaian et al., 1994). A minor divergence exists with respect to Arg 140. A sevenfold increase in the apparent K_i for AMP was found when Arg 140 was mutated to an alanine (Gidhjaian et al., 1994). This increase was correlated to the loss of the hydrogen bond between Arg 140 NH₂ and O3', which was observed in subunit B, C, and D, but not in subunit A (Gidhjaian et al., 1994). In all three structures presented here, only two of four subunits contain this hydrogen bond. In addition, there is no consistency in terms of which subunit contains the hydrogen bond: subunit C and D in the FB:AMP complex, subunit A and B in the FB:FMP complex, and subunit A and D in the FB:AICAR-P complex (Table 3). The Arg 140 side-chain conformation is not restricted to a common conformation in any of the structures, and thereby not favoring a hydrogen bond to O3'. Furthermore, if the Arg 140 NH₂ to O3' hydrogen bond is important in ligand binding or activation of the allosteric mechanism, removal of the 3-hydroxyl group of AMP will be expected to induce the same order of reduction in inhibitory activity. However, the 3-deoxy-AMP was found to be a more potent inhibitor of human FB than AMP (Table 4). These findings imply that the particular hydrogen bond is insignificant. The mutation of Arg 140 to an alanine might trigger some other unknown structural changes, possibly via a long-range electrostatic effect.

Materials and methods

Cloning

Based on the published sequence of the human liver FB (Elmaghrabi et al., 1993), two PCR primers were synthesized that would amplify the coding region of the gene: FB-1, 5'-AAAAAAGGATCCAGCATGGCTGACCAGGCGCCC-3', introducing a *Bam*H I site in front of the AUG start codon; FB-2, 5'-AAAAAAGCTTCAGGTGCTCACTGGGCAGAGTGC-3', introducing a *Hind* III site overlapping the UGA stop codon. Human liver poly-A⁺ RNA was purchased from Clontech (cat. 6510-1, lot #39076). First strand cDNA was made and this was used as template for the PCR reaction: one cycle 95 °C/30 s, 65 °C/30 s, 72 °C/180 s, followed by 20 cycles 95 °C/30 s, 72 °C/150 s. After the PCR reaction, the DNA was cut with *Bam*H I and *Hind* III restriction enzymes and the fragment of about 1,000 bp was isolated from an agarose gel. The fragment was inserted into the vector pBlueBac III (Invitrogen) and cut with the same enzymes, giving rise to plasmid pSX564. This can be used for expression of the FB enzyme in insect cells

Fig. 5. Atomic numbering for each inhibitor and the definition of torsion angles examined in the grid search.

using the Baculovirus system. The sequence of the cDNA was the same as that found by Elmaghrabi et al. (1993), except that CG in position 786–787 should be GC, which brings the protein sequence in accordance with the other enzymes in their own alignment—Gly instead of Ala in position 214. This is also what is found in the cDNA cloned from the human leukemia cell line HL60 (Solomon et al., 1988).

Transfection and isolation of recombinant viruses

The cloning vector, pBlueBacIII, *Autographica californica* nuclear polyhedrosis virus (AcMNPV) DNA, *Spodoptera frugiperda* (SF9)

cells, Baculovirus agarose, Grace media + supplements, and Cationic liposome solution were from Invitrogen. Fetal calf serum (FCS) was from GIBCO BRL/Life Technologies.

Transfection was performed as described in the Baculovirus Expression System Manual from Invitrogen (version 1.5.5). One microgram of linear AcMNPV DNA and 3 μ g of transfer plasmid were transfected into SF9 cells (2×10^6 cells in 60 mm dishes) seeded in complete Grace medium (Grace medium + supplements + 10% (v/v) heat inactivated FCS). The resulting culture supernatant was collected after five days. Fresh monolayers of SF9 cells (6×10^6 cells in 100-mm dishes) were infected with virus supernatant at various dilutions and then overlaid with 1.5% agarose containing complete Grace medium with 150 μ g/mL X-gal. After six days,

presumed recombinant plaques were identified by their blue color and used to infect 6-well plates containing SF9 cells. After five days, the corresponding virus DNA was purified and subjected to a PCR reaction with forward and reverse primers as described previously (Malitschek & Scharl, 1991). Recombinant viruses were subjected to another round of plaque purification to ensure that the recombinant virus stock was free of wild-type virus.

Expression and purification

The infected cells were maintained in complete Grace medium at 27°C in suspension culture. The cells in logarithmic phase (originating from 100 mL of SF9 cell culture) were isolated by centrifugation, and the cell pellet homogenized at 4°C (Polytron) in 2.5 mL of 50 mM HEPES buffer, pH 7.5, containing 5 mM MgSO₄, 0.1 mM EDTA, and 1 mM DTT. After extraction, the sample was centrifuged at 15,000 rpm for 10 min.

Ten milliliters of 10 mM MES buffer, pH 6.0, containing 0.1 mM EDTA and 5 mM DTT (A-buffer) were added to the turbid supernatant. The resulting solution had a conductivity of 1.3 mS and was still turbid. The solution was centrifuged at 4,000 rpm for 15 min. To the resulting solution was further added A-buffer, to a total volume of 28 mL. The resulting solution was pumped onto a HiLoad 16/10 (Pharmacia) SP-Sepharose column (1.6 × 10 cm) at a flow rate of 6 mL/min. Previous to the application, the column was equilibrated with A-buffer.

The column was washed with 50 mL A-buffer, and FB was eluted with a linear gradient between A-buffer and A-buffer containing 1 M KCl (B-buffer) and had a conductivity of 195 mS. The gradient was applied from 0% B-buffer to 50% B-buffer over 100 min at a flow rate of 6 mL/min. Fractions corresponding to 6 mL were collected, and the absorbance at 280 nm was recorded (AUFS = 0.5). FB eluted as a symmetrical peak at a position corresponding to 0.16 M KCl. Five fractions containing the active enzyme were pooled.

The FB purified by the above method ends up in a solution too dilute for crystallization experiments (protein concentration: 58 µg/mL). Therefore, the sample was concentrated using the following procedure: 10 volumes of 10 mM MES buffer, pH 6.0, containing 1 mM EDTA and 1 mM DTT, were added to the enzyme pool (30 mL) from the HiLoad column, and the resulting solution (330 mL) was pumped onto a Mono S column (1 mL) (Pharmacia) equilibrated previously with A-buffer. After application, the column was washed with 10 mL of A-buffer, and the FB was eluted by a step gradient from A-buffer to A-buffer containing 0.3 M KCl. Using this procedure, the protein was eluted in a volume of 0.3–0.5 mL with a protein concentration of 3–6 mg/mL. A total yield of 1.74 mg highly purified and concentrated recombinant FB was obtained from 100 mL SF9 cell culture. The specific activity of FB was 2.4 ± 0.1 (SD) units/mg protein (data not shown).

Crystallization

After the last purification step, the enzyme was washed 3–4 times with C-buffer containing 20 mM Tris-base buffer, pH 7.5, 2 mM

Fig. 6. Central water molecule in the allosteric binding pocket of the three complexes and the possible hydrogen bonds between the central water molecule and the ligand and the protein, respectively. Average hydrogen bond lengths are given in the drawing.

Fig. 7. Superimposition of the inhibitors in which all the atoms of the ribose rings are fitted together. **A:** Three inhibitors from the FB complexes of the present study. **B:** AICAR-P from the FB:AICAR-P complex and AICAR-P from the CSD. **C:** AMP and FMP from the FB:AMP and FB:FMP complexes and the AICAR-P from CSD. **D:** AMP, FMP, AICAR-P from the FB:AMP, FB:FMP, and FB:AICAR-P complexes and AICAR-P (CSD) from a side view. Atoms are colored according to atom type (carbon and phosphorus, yellow; oxygen, red; nitrogen, blue).

MgSO₄, 0.1 mM EDTA, 1 mM DTT, and 5% glycerol using Microsep Microconcentrators 30 K. In the last washing step, 3 mM of either AMP, AICAR-P, or FMP were added to C-buffer. The enzyme was concentrated to 10 mg/mL on MicrosepTM microconcentrators (Filtron Technology Corporation).

Crystals of FB:AMP, FB:AICAR-P, and FB:FMP complexes were grown by dialyzing 10 mg/mL enzyme solution against D-buffer,

consisting of 50 mM Tris-base buffer, pH 8.4, 0.1 mM Li₂SO₄, 0.1 mM sodium citrate, 0.1 mM EDTA, 5 mM NaN₃, 1 mM mercaptoethanol, 6.5–8% w/v PEG (*M_r* 3,350), and 3 mM of each inhibitor, respectively (Gidhain et al., 1994). The membrane used was Spectra/Por MWCO: 3,500. The FB:AMP and FB:AICAR-P complexes were grown using dialysis buttons (Cambridge Repetition Engineers) containing 20 μ L enzyme solution. Crystals of typically $0.4 \times 0.2 \times 0.2$ mm grow within 5–7 days of dialysis. The FB:FMP crystals were grown by dialysis in glass tubes with 40 μ L protein solution. Large crystals having a size of $1.0\text{--}0.6 \times 0.4 \times 0.4$ mm were obtained within 10–14 days of dialysis. All crystallization experiments were performed at 18 °C.

Table 4. *IC*₅₀ values of inhibitors

Ligand	<i>IC</i> ₅₀ (μ M) $\pm$ SD	$\times$ AMP level
AMP	1.7 ± 0.1	1.0
AICAR-P	20.9 ± 0.2	12.3
FMP	1.4 ± 0.1	0.83
7-deaza-AMP	>500	—
3-deoxy-AMP	1.4 ± 0.08	0.83

Data collection and data processing

Several days (2–7) before mounting the FB:AMP and FB:AICAR-P crystals, glycerol was added to the external D-buffer. The final glycerol concentration in the external D-buffer was 20%. The FB:FMP crystals could not be mounted without adding extra liquid (mother liquor) to the crystals in the glass tube, because almost all

the liquid was removed by dialysis. If the external D-buffer was added to the crystals, they started to crack. Therefore, the following solution was prepared and added to the crystals: 30% glycerol and 5% w/v PEG 3350 in new mother liquor. The new mother liquor was made as follows: 0.5 mL of C-buffer was dialyzed against 10 mL of D-buffer for two weeks. The membrane used was Spectra/Por MWCO: 3,500. The added solution was also used as a cryoprotectant for the FB:FMP crystals.

The first data set of the FB:AMP complex was collected using a 30-cm Mar imaging plate placed 220 mm from the crystal on a rotating anode X-ray source. The oscillation range was 2° per image, the exposure time was 60 min per image, and the collection was done at room temperature.

In addition, diffraction data were collected on the Hamburg EMBL synchrotron station (X11). Data frames of 0.4–1° oscillations were recorded using a 30-cm MAR imaging plate placed 335 mm (FB:AMP crystal), 400 mm (FB:AICAR-P crystal), and 290 mm (FB:FMP crystal) from the crystals. The exposure time varied from 90 s to 300 s depending on the oscillation range. All Hamburg data were collected at cryogenic temperature (110 K) and only one crystal of each complex was used.

All data were processed using DENZO (Otwinowski, 1986) and the CCP4 suite of programs. Crystal data and statistics of the data collections are given in Table 2.

Structure solution and refinement

The structure of the FB complexed with AMP (room temperature data) was solved by molecular replacement using the program AMORE from the CCP4 package. A monomer of the porcine FB (Ke et al., 1991a) was used as a search model in the resolution range 8–4 Å. In the rotational search, four top solutions came up, corresponding to the tetramer of the human FB enzyme. The translational search was conducted using only the top solution from the rotation search. A second translation search with two models was performed, fixing the first solution to the translation function. This procedure was continued until all four monomers were found. The *R*-factors after each translation search were 53.6%, 50.3%, 48.1%, and 45.8%, respectively, and the correlation coefficients were 16.7, 25.7, 33.6, and 39.5. Ten cycles of rigid-body refinement were performed using AMORE and the *R*-factor dropped to 44.6%. At this stage, the amino acid sequence of the human FB enzyme was introduced to replace that of the porcine FB. The model was inspected using the program O (Jones et al., 1991) and incorrectly placed residues and side chains were adjusted. Only the A monomer was inspected and corrected. The B, C, and D monomers were generated via the noncrystallographic symmetry from the A monomer using the LSQKAB program from the CCP4 package. Three cycles of positional least-squares refinement were performed with PROLSQ from CCP4 and the *R*-factor decreased to 26.9%. All *B*-factors were set to 15 Å². Manual refitting using O in connection with additional 45 cycles with positional and *B*-factor least-squares refinements were done with PROLSQ and the *R*-factor dropped to 22.7%. Water molecules were added to the structures after the following criteria: difference Fourier $|F_o| - |F_c|$ peaks should be stronger than 3σ and defined in the $2|F_o| - |F_c|$ electron density maps contoured at 1σ level, the density should be nearly spherical, satisfactory hydrogen bond interactions should be formed to protein atoms or other water molecules, and the *B*-factor for the waters should be less than 50 Å². Two-hundred seventy-two water molecules were

identified by those criteria and the *R*-factor decreased to 20.2% (Table 2).

The data of FB:AMP were collected at room temperature, resulting in a larger unit cell compared to the synchrotron cryo-data sets. Thus, difference Fourier could not be used to solve the three other data sets. Therefore, the molecular replacement method was used. A refined monomer from the FB:AMP structure (room temperature data) with all flexible and poorly defined residues omitted, was used as a search model. The molecular replacement solution and refinement procedure were almost identical to the protocol described above. All four monomers were refined with several cycles of least-squares refinements using PROLSQ and manual interventions, adjusting incorrectly placed residues and side chains, using O. All three structures were refined following the same protocol.

Assay of FB activity

FB enzyme activity was assayed as the rate of NADPH formation at 25 °C in coupled reaction with phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G-6-P DH). The reaction mixture included 50 mM HEPES, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM NADP⁺, 2 mM β-mercaptoethanol, 1.4 units/mL G-6-P DH, 3.5 units/mL PGI, 100 μM fructose 1,6-bisphosphate, and fructose-1,6-bisphosphatase.

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