Purification and Properties of Oxidized and Reduced Forms of Hepatic
Fructose 1,6-Bisphosphatase*

Ulrich K. Moser†, Marianne Althaus-Salzmann§, Cornelis Van Dop¶, and Henry A. Lardy
From the Institute for Enzyme Research and the Department of Biochemistry, University of Wisconsin,
Madison, Wisconsin 53706

Fructose bisphosphatase is strongly inhibited by its allo-
steric effectors AMP (1-3) and fructose 2,6-bisphosphate (4, 5). The inhibition by AMP poses an enigma, for the enzyme
must function in hepatic gluconeogenesis despite the presence
of AMP concentrations that exceed the K_i (1, 2, 6-8). Fur-
thermore, there is no inverse relationship between cytosolic
AMP concentration and rates of gluconeogenesis (8). The
inhibition is dependent on temperature, pH, and enzyme
concentration (1, 9, 10). Several conditions have been found
to abolish the inhibition; e.g. modification of tryosine (11) or
arginyl residues (12), or treatment with salicylate
(14). These findings provide more information about the sites
influenced by AMP inhibition together with an increase of
activity at alkaline pH (15).

During a study of fructose bisphosphatase we found that
two forms of the enzyme could be prepared from rat liver
cytosol. It appeared that one form could be converted to the
other by reducing agents, and purification of the separate
forms was facilitated by this knowledge. The present study
indicates that a change in the redox state of thiol groups of
the neutral rat liver fructose bisphosphatase is accompanied
by an alteration of the inhibitory effect of AMP.

EXPERIMENTAL PROCEDURES

Purification of Rat Liver Fructose Bisphosphatase—Column frac-
tions were performed at room temperature. All other manipu-
lations of the enzyme, including isoelectric focusing, were carried out
at 4°C.

The livers of 10 female rats (King, retired breeders) were perfused
with ice-cold 0.9% NaCl through the portal vein until free of blood,
inixed in 2 volumes of 0.2 m sucrose, 25 mM K-phosphate, pH 7.5, 1
mM N-trisyl-L-lysyl-chloromethylketone, 1 mM EDTA, homogenized
in a Potter-Elvehjem homogenizer, and centrifuged at 100,000 × g for
60 min. The supernatant solution was then rapidly heated to 65°C
for 3 min and then cooled on an ice-salt mixture. Denatured proteins
were removed by centrifugation at 10,000 × g for 20 min and proteins
in the supernatant solution were separated from the homogenization
medium by gel filtration on Sephadex G-25 equilibrated in 10 mM
K-phosphate, pH 6.0, 0.1 mM EDTA. The enzyme solution was then
divided into two parts of equal volume. To part "A," dithiothreitol
was added to a final concentration of 1 mM, and to part "B," K-
phosphate to a concentration of 75 mM, pH 6.3.

Part A was then applied to a P-cellulose column (3.6 × 22 cm) and
equiluted with 2 mM Fru-P_2 in 10 mM K-phosphate, pH 6.0, 1 mM
dithiothreitol, 0.1 mM EDTA (16). The active fractions were combined
and concentrated by Millipore filtration (immisible CX); equil-
ibrated with 5 mM melsionate, pH 5.8, 1 mM dithiothreitol, 0.1 mM
EDTA by gel filtration; applied to a CM-cellulose column (1.8 × 19
cm) and eluted with 2 mM Fru-P_2 (17-19). The enzyme solution was
concentrated and equilibrated in 10 mM Tris, pH 7.5, 1 mM dithio-
threitol, 0.1 mM EDTA by gel filtration. Glycerol was added to make
a 20% solution and the enzyme (form A) was kept at 4°C.

Part B was then applied to a DEAE-cellulose column (3.6 × 22 cm)
equiluted with a 1-liter linear gradient of 40-500 mM K-
phosphate, pH 6.3, containing 0.1 mM EDTA and 2.0 mM Fru-P_2 (19).
After concentrating and equilibrating in 10 mM K-phosphate, pH 6.3,
and 0.1 mM EDTA by gel filtration, glycerol and ampholines (pH 5-7)
were added to make 20% and 1% solutions, respectively. Isoelectric
focusing was performed in a 110-ml tube using a 5-60% glycerol
gradient and ampholines, pH 5-7, with 0.1 mM EDTA. After 24 h at
1200 volts, the column was eluted slowly using a Gibson pump (20).
Fractions with enzyme activity were combined, concentrated, and
applied to a G-200 column equilibrated with 10 mM Tris, pH 7.5, 0.1
mM EDTA. The fractions with the highest specific activity were
combined, concentrated, and stored at 4°C in 20% glycerol. A com-
bination of both isoelectric focusing and a G-200 column had to be
used following the DEAE-cellulose step, since neither applied alone
purified the enzyme sufficiently so as to yield a single band on SDS
gels. A typical purification of the two forms of fructose bisphosphatase
is summarized in Table I.

The isolation of both forms A and B was later found to be
significantly improved by using blue dextran-Sepharose, as introduced

* Supported by Grants AM20678 and AM10,334 from the National
Institutes of Health. The costs of publication of this article were
defrayed in part by the payment of page charges. This article must
therefore be hereby marked "advertisement" in accordance with 18
U.S.C. Section 1734 solely to indicate this fact.
† Postdoctoral Fellow of the Swiss National Science Foundation.
‡ Present address, Hoffmann LaRoche, CH4002, Basel, Switzerland.
§ Present address, University of California Medical School, De-
partment of Pediatrics, San Francisco, CA 94121.
Oxidized and Reduced Forms of Fructose 1,6-Bisphosphatase

Fructose 1,6-bisphosphatase (Fr-1,6-Pase) was prepared and assayed as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Step</th>
<th>Fr-1,6-Pase</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Inhibition by AMP (µM)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2880</td>
<td>8230°</td>
<td>0.4</td>
<td>100</td>
<td>88 98</td>
</tr>
<tr>
<td>Heat</td>
<td>2350</td>
<td>1280°</td>
<td>1.0</td>
<td>81</td>
<td>74 94</td>
</tr>
<tr>
<td>Part &quot;A&quot;:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-Cellulose</td>
<td>480</td>
<td>6,4°</td>
<td>7,5</td>
<td>33</td>
<td>90 95</td>
</tr>
<tr>
<td>CM-Cellulose</td>
<td>150</td>
<td>1,8°</td>
<td>93</td>
<td>10</td>
<td>88 97</td>
</tr>
<tr>
<td>Part &quot;B&quot;:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>610</td>
<td>33,7°</td>
<td>18</td>
<td>42</td>
<td>56 87</td>
</tr>
<tr>
<td>IEF</td>
<td>200</td>
<td>3,0°</td>
<td>65</td>
<td>13</td>
<td>29 79</td>
</tr>
<tr>
<td>G-200</td>
<td>85</td>
<td>1,2°</td>
<td>71</td>
<td>6</td>
<td>28 79</td>
</tr>
</tbody>
</table>

* The yields in part "A" and "B" were calculated as if all, rather than half of the crude extract had been processed by each procedure. 

**AMP (µM) was added to the assay mixture.

Table 1

RESULTS

Both forms of the enzyme, purified in the presence or absence of dithiothreitol, exhibited a single band on SDS-polyacrylamide gels (Fig. 1A), whether the isolation had been performed according to Table I or with blue Sepharose. Scanning the gels revealed a purity of 95–100% for both forms. In addition, gel filtration on a G-200 column resulted in the same elution volume (not shown). The estimated molecular weight of 37,000/30,000 (Fig. 1B) for either form was in accordance with data of Tejwani et al. (17) for rat liver fructose bisphosphatase isolated in the presence of β-mercaptoethanol. Form A and B exhibited equal affinity for the substrate (Fig. 2), and the K	extsubscript{m} of 2 µM was in good agreement with data of Taketa and Pogell (1), Tashima et al. (22,23), or Tramiello (16). However, AMP inhibited form A about three times more effectively than form B (Fig. 3), with the greatest differences between 200 and 300 µM AMP. The K	extsubscript{i} of 140 µM for form A was of the same order as is reported by Taketa and Pogell (1); that of form B was increased to 370 µM. The slopes of the Hill plots were about 2 for both A and B as in (17), indicating an unchanged number of binding sites.

In order to determine whether the stronger inhibition by AMP of form A was solely due to the action of dithiothreitol, aliquots of form A and B were incubated in the presence and absence of 1 mM dithiothreitol at 37 °C for 0–60 min. At various time intervals, samples were assayed for enzyme activity and AMP inhibition (Fig. 4). The percentage of inhibition by 250 µM AMP in the absence of dithiothreitol remained constant for both forms; but in the presence of 1 mM dithiothreitol the sensitivity of enzyme B to AMP increased until it equaled that of enzyme A. The retained high level of AMP inhibition of form A in the absence of dithiothreitol might have been explained by the dithiothreitol still present from the storage buffer of the enzyme. However, passing the enzyme over a Sephadex G-25 column to remove all the dithiothreitol did not change the high sensitivity of the enzyme to AMP. With some preparations of enzyme B exhibiting specific activities of less than 100, incubation with dithiothreitol occasionally increased the activity by about 30%. The specific activity did not, however, reach that of enzyme A.

Since dithiothreitol, a dithiol reducing and protecting agent (27), could increase the sensitivity of form B to AMP, the thiol groups of form A and B of the enzyme were investigated. Enzyme preparations from blue Sepharose were desalted on G-25 columns, whereby no dithiothreitol could be detected in the filtrate of the last concentration step. The total number of thiol groups was found to be 22.4/enzyme in form A and 19.2 in form B (2 determinations). The calculations were based on Coomassie blue protein quantitations with bovine serum albumin as standard and an estimated molecular weight of 150,000.

Form A and B also differed in the reactivity of their thiol groups. In form A, about 50±13 thiol groups/enzyme were reactive very rapidly with DTNB, whereas only 0.5 fast reacting groups could be observed with form B (Fig. 5). The difference measured corresponded to one pair of vicinal thiols in A having been oxidized to the disulfide state in B. AMP induced an inhibition of thiol reactivity initially but enhanced the total number of thiols that reacted during a 10-min reaction period.

The same pattern of thiol reactivity was found in the 50 mM Tris-HCl, pH 8.0, medium (see "Experimental Procedures,"...
Oxidized and Reduced Forms of Fructose 1,6-Bisphosphatase

PART A

FIG. 1. SDS-gel electrophoresis of fructose bisphosphatase. 10 µg of enzyme A or B were applied on 5% acrylamide gels. After electrophoresis, the gels were scanned in a Beckman spectrophotometer with attached microprocessor. With either enzyme A or B, 95–100% of the total area was found under the peak. A, photograph of a gel with enzymes A and B. B, molecular weight determination from standard proteins (Kit combithek, Boehringer Mannheim).

FIG. 2. Effect of substrate concentrations on fructose bisphosphatase activity. Enzyme A (○) and enzyme B (▲) were assayed using the Fru-P₂ regenerating mixture as described under Experimental Procedures. The inset shows the Lineweaver-Burk plot of the data of this figure for the range of noninhibitory substrate concentrations. 100% relative activity is 100 units/mg for enzyme A and 60 units/mg for enzyme B.

FIG. 3. Inhibition fructose bisphosphatase by AMP. Aliquots of enzyme A (○) or enzyme B (▲) were assayed in the presence of varying concentrations of AMP as described under Experimental Procedures. The reactions were started by adding 50 µM Fru-P₂. The inset shows a Hill plot of the data. V_{max} was the relative enzyme activity without AMP, v the activity in the presence of AMP, n is the slope of each line, and K, the concentration of AMP necessary for 50% inhibition. 100% relative activity was 100 units/mg for enzyme A and 60 units/mg for enzyme B.

Has been reported for rat liver fructose bisphosphatase (30). This form of the enzyme could be phosphorylated by the catalytic subunit of protein kinase, whereas the trypsin-treated enzyme with a molecular weight of 36–37,000 was not phosphorylated. Since different molecular weights for the same preparation of fructose bisphosphatase had been obtained with different SDS-polyacrylamide gel electrophoresis systems (31), the molecular weight of our enzyme preparation was rechecked by a second procedure. In the 10% (w/v) SDS-acrylamide slab gel electrophoresis system (10 × 0.15 cm) according to Laemmli (32) with ovalbumin (43,000), aldolase (40,000), lactate dehydrogenase (35,000), and chymotrypsinogen A (25,000) as standards, it was found to be essentially the
modified by proteolytic enzymes during purification. Although the activity of the “neutral” enzyme was also shown to be enhanced by incubation with cystamine or p-chloromercuribenzoate under certain conditions (28, 35), enhancement of activity at neutral pH by sulfhydryl reagents is not commonly considered as a major physiological mechanism.

Only limited data are available on the effect of sulfhydryl reagents on AMP inhibition and these have been obtained with enzymes exhibiting a pH maximum in the alkaline range. p-Mercuribenzoate abolished the AMP inhibition in fructose bisphosphatase of candida utilis (36), whereas disulfide forming reagents and p-chloromercuribenzoate enhanced it in rabbit liver fructose bisphosphatase (33, 37).

The purpose of these studies has been to investigate the possible function of sulfhydryl groups of the neutral fructose bisphosphatase in the inhibition exerted by AMP. Procedures have been developed to isolate reduced and oxidized forms of the enzyme. The shortest isolation time, best yield, and specific activity were achieved with the isolation procedure comprising a DEAE-cellulose and a blue Sepharose affinity chromatography step. The 2 forms of the neutral enzyme isolated in the presence and absence of dithiothreitol have equal molecular weights, affinity for Fr-P, and high substrate inhibition, but differ in their sensitivity to the allosteric modulator AMP. Inhibition by AMP of form B is diminished to approximately ½ that of form A, with the Kf for AMP shifted from 140 to 370 mM. When form B was reduced with dithiothreitol, it regained sensitivity to AMP inhibition equivalent to that of form A.

The conclusion that the difference between forms A and B is in the state of oxidation of sulfhydryl groups has been confirmed by determinations of the number and reactivity of these groups. The total of 22.4 sulfhydryl groups/enzyme measured in form A is comparable with the 6 to 7 carboxymethyl cysteine residues/subunit measured in Ref. 17; 3.2 thiol groups less have been found in form B. The striking difference in fast reacting groups of forms A and B of 2.5 and 0.5, respectively, suggests that these thiol groups must be functional for the maximal inhibitory effect of AMP. Conclusions about the number of thiol groups per enzyme functional for the AMP inhibition cannot, however, be drawn from these data, since the measurements have not been designed to solve this question.

Because the extent of inhibition by AMP after the heat step and the G-25 column purification is similar to that obtained with the crude extract, and yet most of the purified enzyme isolated in the absence of dithiothreitol exhibits a low AMP inhibition, it might be speculated that a heat stable protein such as, e.g. thioredoxin, has kept the enzyme reduced during the initial isolation steps. After the first adsorption column, this molecule might have been removed from fructose bisphosphatase as indicated by the decline of AMP inhibition in the absence of dithiothreitol. The system ferredoxin/thioredoxin regulates the chloroplast fructose bisphosphatase (38) and a combination of thioredoxin, thioredoxin-reductase, and NADPH is able to reduce insulin. However, an attempt to use the latter system for the regulation of enzyme B has not been successful.

Fructose bisphosphatase from rat liver can be phosphorylated (19, 30, 39) and it is postulated that this might be part of a regulation mechanism. Our enzyme preparation is slightly proteolyzed in that it has a lower molecular weight than the most recently published one (30) and is missing the phosphorylatable protein fragment of the carboxyterminal sequence. If evidence for the existence of the two forms in vivo is eventually obtained, it will be interesting to find out how phosphorylation and the newly discovered modulator of fruc-
Oxidized and Reduced Forms of Fructose 1,6-Bisphosphatase

tose bisphosphatase, fructose 2,6-phosphate, which synergistically enhances AMP inhibition (4, 5) are involved in our proposed redox mechanism.

Acknowledgment—We thank Madeleine Moser for excellent technical assistance.

REFERENCES

**Purification and properties of Oxidized and reduced forms of Hepatic fructose 1,6-bisphosphatase.**

U K Moser, M Althaus-Salzmann, C Van Dop and H A Lardy


Access the most updated version of this article at [http://www.jbc.org/content/257/8/4552](http://www.jbc.org/content/257/8/4552)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/257/8/4552.full.html#ref-list-1](http://www.jbc.org/content/257/8/4552.full.html#ref-list-1)