Amino Acid Sequence of Rabbit Muscle Aldolase and the Structure of the Active Center

Abstract. *Elucidation of the amino acid sequence of fructose-1,6-bis-phosphate aldolase from rabbit muscle has made it possible to assign the positions of the functional groups known to play specific roles in the catalytic activity, and also to locate the buried, exposed, and active site cysteine residues. The results indicate that the middle portion of the polypeptide chain, including Cys-134, Cys-149, Cys-177, and Cys-199, is buried in the native structure, with regions containing Cys-72, Lys-107, Lys-227, Cys-336, His-359, and the COOH-terminal residue (Tyr-361) folded into the active center of the enzyme, at or near the surface of the enzyme molecule.*

Fructose-1,6-bis-phosphate aldolase catalyzes a key reaction in glycolysis and energy production, namely, the reversible cleavage of fructose 1,6-bisphosphate ($FruP_2$) to form glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The enzyme constitutes 3 percent of the soluble protein in rabbit muscle, and crystalline preparations from this source have been the subject of numerous studies on the relation of structure to catalytic function. A number of functional groups have been identified and shown to play specific roles in the aldol cleavage reaction (*1*). We now report the elucidation of the complete primary structure of the enzyme (*2*), and the location in this sequence of the functional groups, as well as of the eight cysteine residues. These cysteine residues are known to differ widely in reactivity toward a variety of sulfhydryl reagents (*3*, *4*). This information has provided clues to essential features of the active center and the three-dimensional folding of the polypeptide chain, and is important for x-ray crystallographic studies of the tertiary and quaternary structure (*5*).

Rabbit muscle $FruP_2$ aldolase is a protein (molecular weight 160,000) composed of four subunits (*6*). The tetrameric structure was confirmed by experiments in which four peptide fragments were obtained by cleavage of the 12 methionyl bonds in the enzyme with BrCN (*7*). It has thus been established that each subunit in the molecule contains three methionine residues located at identical positions, yielding four unique BrCN peptide segments, and that the subunits are very similar, if not identical. The largest of the four BrCN peptides was found to derive from the NH_2-terminal portion of the subunit chain (peptide N), the second largest from the COOH-terminus (peptide C). The two smaller peptides (peptides A and B) were shown to be linked in that order, occupying the center portion of the molecule (*7*). This finding greatly facilitated the study of the total primary structure of rabbit muscle aldolase, since the enzyme could be fragmented into peptides of manageable size (molecular weights ranging from 2,000 to 18,000) and the sequence analysis could be carried out with each fragment. The amino acid sequences of peptides A and B, including the sequence around the active site lysine residue (*8*), have been reported (*9*).

Earlier findings that aldolase was composed of nonidentical subunits (*10*) were confirmed by the separation of α and β subunits of differing electrophoretic mobility and susceptibility to carboxypeptidase digestion (*11*, *12*). However, peptide mapping of the corresponding BrCN peptides obtained from the separated α and β subunits revealed only minor differences, confined to a small region in the peptide C segment (*12*), and structural analyses of two COOH-terminal peptides isolated from the peptide C segment showed that an asparagine residue in the α chain was replaced by aspartic

Fig. 1. The amino acid sequence of the monomer of rabbit muscle fructose-1,6-bis-phosphate aldolase. The following amino acid residues are involved in the indicated functions: Lys-227, formation of Schiff base with substrate; Lys-107, secondary binding of substrate; Cys-72 and Cys-336, formation of a disulfide bridge, one of them involved in the aldol cleavage; His-359, required for proton exchange; Tyr-361, required for enhanced cleavage of $FruP_2$. Cys-134, Cys-149, Cys-177, and Cys-199 are buried, whereas Cys-72, Cys-237, Cys-287, and Cys-336 are exposed in the native enzyme. Of four glutamic acid residues at positions 272 to 275 one is probably glutamine (*2*). Abbreviations for the amino acid residues are: Ala, alanine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Trp, tryptophan; Val, valine.

acid in the β chain (*13*). Deamidation of this asparagine residue (Asn-358) has now been confirmed as the only difference between the α and β subunits; no other structural differences were encountered in the sequence analysis of the other BrCN peptides (*2, 9, 14*). The proposed primary structure of the muscle aldolase subunit is shown in Fig. 1 (*2*).

Information from many laboratories, including our own, on the mode of action of $FruP_2$ aldolase and the role of functional groups in the catalysis is summarized in the scheme shown in Fig. 2 (*1*). Initial interaction of $FruP_2$ with the enzyme involves primarily electrostatic forces between the phosphate group at C-1 of the fructose and an as yet unidentified, positively charged group at the active center (*15*) (step 1). A weaker electrostatic interaction involves the C-6 phosphate group of the substrate and a second lysine residue at the active center (see below). Formation of the enzyme substrate complex (step 1) is followed by elimination of a mole of water between the carbonyl group of $FruP_2$ and the ϵ-amino group of Lys-227 to form the catalytic Schiff base intermediate (*8, 9*) (step 2). The protonated Schiff base intermediate, in the ketimine form, promotes a shift of electrons that labilizes the bond between C-3 and C-4. Cleavage of this C-C bond (step 3) is also facilitated by the withdrawal of a proton from the C-4 hydroxyl group of the substrate by the conjugate base formed when the cysteine sulfhydryl group loses a proton to Lys-107, an event promoted by the interaction of this lysine residue with the C-6 phosphate group (step 1). This role for Lys-107 is based on its modification by pyridoxal phosphate in the absence of substrates, with concomitant loss of catalytic activity (*16*).

We have also identified a pair of essential cysteinyl residues at the active site (Cys-72 and Cys-336), which are readily oxidized to form a disulfide bridge, with loss of catalytic activity (*17*). One of these cysteine residues is shown to undergo an abortive reaction with the substrate, glyceraldehyde 3-phosphate (*18*). After release of the glyceraldehyde-phosphate moiety in step 4 of the reaction, protonation and hydrolysis of the dihydroxyacetone Schiff base takes place, probably mediated by a histidine residue at the active center (*19*). This function of histidine in the reaction mechanism was deduced from photooxidation experiments in which inactivation of the enzyme paralleled the destruction of histidine, with loss of the proton exchange reaction (*19*). Three histidine residues are located near the active site lysine at positions 217, 218, and 235; but the last has been excluded from a functional role because it is replaced by asparagine in the active site peptides of frog and lobster muscle aldolases and by glutamine in aldolase of sturgeon muscle (*20*). Hartman and Welch have used a specific alkylating agent to show that His-359 is the functional residue (*21*). This is of particular interest, since it is near the COOH-terminal tyrosine residue (Tyr-361), long recognized as essential for the enhanced activity of the enzyme toward $FruP_2$ as compared with fructose 1-phosphate (*10, 22*). The evidence suggests that this tyrosine is required to maintain the structure that enables His-359 to function in proton transport to the Schiff base carbanion, and is also essential for the interaction of Lys-107 with Cys-72 (or Cys-336) (*1, 22*).

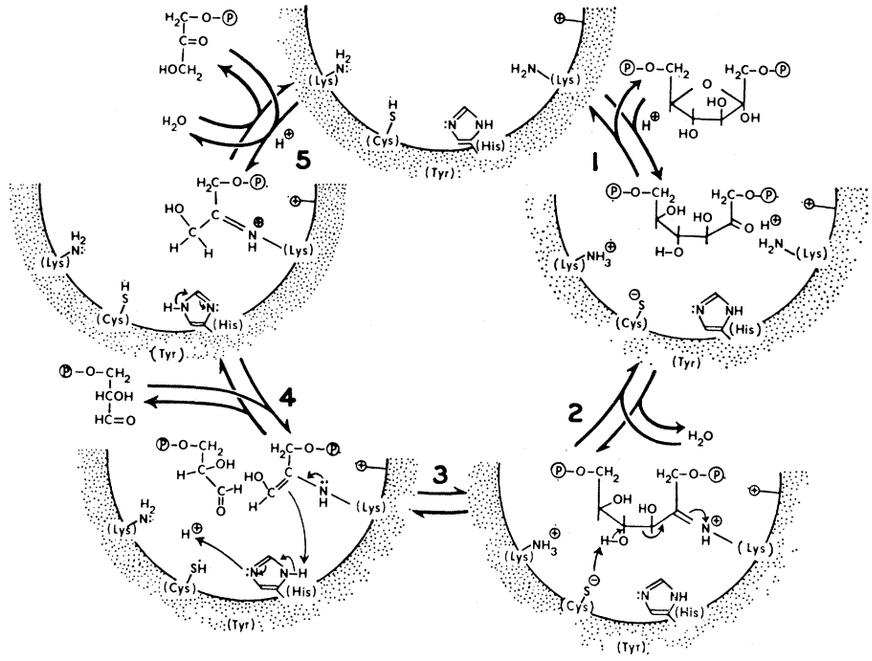

Fig. 2. Mechanism of action of rabbit muscle aldolase. The functional groups shown are, from left to right: Lys-107, Cys-72 or Cys-336, Tyr-361, His-359, and Lys-227.

In addition to the functional groups discussed above, the location of all the cysteine residues has now been determined. The four residues found to be readily reactive with a disulfide monosulfoxide reagent (*4*) are Cys-72 and the three residues (Cys-237, Cys-287, Cys-336) located on the COOH-terminal side of the Schiff base–forming lysine residue (Lys-227). The other four cysteine residues, located between positions 134 and 199 are "buried" in the native enzyme since they react with sulfhydryl reagents only after denaturation of the enzyme (*4*). As was mentioned earlier, Cys-72 and Cys-336, located near the two ends of the polypeptide chain, form a disulfide bridge on mild oxidation with an *o*-phenanthroline-Cu^{2+} complex (*17*). This oxidation is prevented by the presence of substrate. The reaction of these cysteine residues with the disulfide monosulfoxide reagent is also blocked by the substrate, supporting the conclusion that they are at the active site (*17*).

These studies have provided information as to the three-dimentional structure of rabbit muscle $FruP_2$ aldolase. The middle third of the subunit, approximately from residue 110 to residue 220, appears to be buried in the native structure of the enzyme. A region including Cys-237 and Cys-287, though close to the active site, lies on the surface of the enzyme. Finally, Cys-72 and Lys-107 at one end of the polypeptide chain, Lys-227, and Cys-336, His-359, and Tyr-361 at the other end must be held in sufficient proximity to form the active center of the enzyme, near the surface of the molecule.

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Diarrhea Toxin Obtained from a Waterbloom-Producing Species, Microcystis aeruginosa Kützing

Abstract. *A diarrhea-producing toxin from a blue-green alga,* Microcystis aeruginosa *Kützing, was obtained from standing laboratory cultures. The nondialyzable fraction of the lysate from whole cells produced fluid accumulation in the ligated small intestinal loops in guinea pigs.*

Algal species have been suspected of producing toxin since 1878 (*1*), and three different types of toxin were obtained from several genera of the Phylum Cyanophyta commonly known as the blue-green algae (*2*). Pathological changes in the liver, heart, lungs, and kidney due to the effects of the extracts from *Microcystis aeruginosa* Kützing, a member of the phylum Cyanophyta, were also reported (*3*). Although no diarrheagenic toxin has been isolated previously from any species of the phylum Cyanophyta (*4*), I have pointed out the importance of further studies on toxic phytoplankton as a cause of gastroenteritis when no known etiological agent could be identified (*5*).

A diarrhea toxin obtained from *Microcystis aeruginosa* laboratory cultures has now been isolated and assayed. Since *Microcystis* is widely distributed in many parts of the world in freshwater sources used by man, domestic animals, and wildlife, the findings reported may have a bearing on their health.

Initial studies with centrifuged waterbloom consisting predominantly of *Microcystis aeruginosa* obtained from a city pond in Dacca, Bangladesh, demonstrated a lethal factor, which was assayed by intraperitoneal injection in rats; but these crude preparations were inconsistent in causing fluid accumulation in the mammalian small intestine. Subsequently a *Microcystis* clone was established from a single cell cultured from a suspension of disrupted colonies separated by the extinction dilution method. The disruption of the colonies was effected by a cell disruption bomb (*6*) at 1000 pounds per square inch. *Microcystis* cells were cultured in one liter of a synthetic, dialyzable aqueous media in 4-liter flasks at 27° ± 1°C for 30 days in low light. *Microcystis* cells were concentrated by the cell disruption bomb, at 500 pounds per square inch for 20 minutes, followed by slow equilibration to atmospheric pressure. Virtually clear fluid was taken from the top, and usually 10^8 *Microcystis* cells per milliliter remained at the bottom of the bomb. The concentrated cell suspension was then frozen and thawed five times and broken by shaking in a flask with glass beads. This suspension was then centrifuged, and the supernatant was sterilized by passing it through a series of Millipore filters. The sterile filtrate was then concentrated 43-fold by vacuum dialysis and reconstituted with distilled water to a concentration tenfold that of the original. This preparation was sterilized again by Millipore filtration and the osmolarity was 11.7 milliosmoles per liter.

This preparation was assayed in ligated intestinal loops of guinea pigs essentially by the described technique (*7*). Modifications included the use of five loops per animal and harvesting at 6 hours. The dialyzable component (24.3 milliosmoles per liter) was assayed in the same way. A preparation of cholera toxin (*8*) containing 278 Lb units or 133 Ll units per milliliter (*9*) was used as the positive control, and the medium

Table 1. Fluid accumulation in the ligated small intestine loops of guinea pigs as a result of the action of the nondialyzable component of *Microcystis* whole cell lysate.

Injection into loop (ml)	Averages of three small intestine loops in three guinea pigs		
	Length of loop (cm)	Fluid accumulation per loop (ml)	Fluid per centimeter (ml)
	Microcystis *toxin*		
2	11.9	3.9	0.33
1	10.2	2.5	0.24
0.5	8.8	1.2	0.14
	*Cholera toxin**		
1	11.2	4.2	0.38
	Growth media for Microcystis		
2	8.0	0	0

* Gel borate buffer containing 0.01 ml (2.78 Lb units) of cholera toxin.

Table 2. Fluid accumulation in the ligated small intestine loops of guinea pigs as a result of the action of the dialyzable component of *Microcystis* whole cell lysate. Cholera toxin was used as a control.

Injection into loop (ml)	Averages of two small intestine loops in two guinea pigs		
	Length of loop (cm)	Fluid accumulation per loop (ml)	Fluid per centimeter (ml)
	Cholera toxin in 2 ml of buffer		
0.01	11.2	4.6	0.42
0.005	8.1	2.6	0.36
0.001	9.5	3.0	0.31
	Dialyzed Microcystis *cell lysate*		
3	14.5	0.1	0
	Growth media for Microcystis		
2	6.4	0	0