Catalytic and Immunoochemical Properties of Homomeric and Heteromeric Combinations of Aldolase Subunits* 

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EDWARD E. PENHOET† AND WILLIAM J. RUTTER‡

From the Department of Biochemistry, University of Washington, Seattle, Washington 98105

SUMMARY

The catalytic and immunoochemical properties of homomeric and heteromeric rabbit fructose diphosphate aldolase tetramers are described. Heteromers composed of either A and C (A&C, A&!, AC) or B and C (B&C, B&, BC) subunits have maximal velocities and Michaelis constants indistinguishable from those of an equivalent mixture of homomers. These results indicate the subunits act independently and also imply the existence of four catalytically active sites in the aldolase molecule (one active site per subunit).

Specific noncross-reaction antibodies prepared against the homomeric aldolases A*, B*, and C* react with heteromers containing one or more subunits to which the antibodies had been directed. This interaction results in essentially complete inhibition of the catalytic activity of the heteromer. The amount of antibody required is inversely proportional to the number of antigenic subunits in the heteromer. These results suggest that (a) interaction with antibody may alter subunit interactions that are required for catalytic activity, and (b) antigenic sites may be modified in homomeric and heteromeric combination.

Many properties of the parental (homomeric) aldolases A4, B4, and C4 have been described (2) and the probable metabolic roles of two of these (A4 and B4) have been delineated (3, 4). In order to understand the physiological significance of multiple aldolases, however, the properties of hybrid (heteromeric) aldolases must also be examined, since in many tissues such heteromers predominate and are presumably responsible for most of the aldolase activity (5-8). In addition to defining metabolic roles, a study of the parental and hybrid aldolases might provide some insight into the effects of homologous and heterologous subunit interactions on the catalytic and structural features of the subunits, a point which is relevant to theories of control of oligomer activity.

We have undertaken an analysis of the AC and BC hybrid sets (heteromers) in conjunction with a previously reported study (2) of the aldolases A, B, and C. The catalytic properties of these homomers are sufficiently different from each other so that significant differences in the properties of the subunits in homomeric versus heteromeric combination would be detected. The data indicate that the catalytic activities of the subunits are not significantly altered in heterologous association. The kinetic properties of heteromers are readily explained by a random combination of individual subunits, each retaining the same catalytic characteristics found in the homomers. Since the molecules are tetramers, these results also imply the existence of four statistically equivalent catalytic sites in aldolase. Previous studies had suggested only three sites (9-11). Interaction of the heteromeric aldolase with antibodies specifically directed against individual homomers showed that the activity of aldolase heteromers can be inhibited by antibodies prepared against either parental species. This finding is most readily interpreted in terms of modulated subunit interactions.

EXPERIMENTAL PROCEDURE

The assay procedures and methods of preparation of aldolases A, B, and C have been reported (12). Antibodies were prepared against rabbit aldolases A, B, and C as previously described (2). The antibody preparations specifically inhibited and precipitated the enzyme to which they were directed and did not cross-react with the other homomeric aldolases (2). The AC hybrid set was isolated from rabbit brain by the procedure of Penhoet, Kochman, and Rutter (2, 12). The BC aldolase hybrid set utilized in these studies was obtained in the following manner: Aldolase B and C were dissolved at 1 mg per ml in 0.01 M Tris-Cl, 0.001 M EDTA, pH 7.5. The solution (46 ml) was acidified to pH 2.2 by the addition of 2 ml of 1 N H3PO4 and was allowed to stand for a period of approximately 10 min at 0°. It was then neutralized at 0° by dilution into 500 ml of 0.01 M Tris-Cl, 0.001 M EDTA, 5 × 10-3 M β-mercaptoethanol, 20% sucrose, pH 8.0, containing a sufficient quantity of 1 M Tris base (2 ml) to neutralize the phosphoric acid. The BC hybrid set was then concentrated in an Amicon ultrafiltration cell to approximately 5 mg of protein per ml. The recovery of FDP cleavage activity during this procedure was 85%. The BC hybrid set produced in this manner was resolved by DEAE-Sephadex A-50 chromatography in a manner similar to that...
FIG. 1. DEAE-Sephadex A-50 resolution of the aldolase BC hybrid set. The BC hybrid set (70 mg) obtained as described under "Experimental Procedure," was equilibrated with 0.05 M Tris-Cl, 0.004 M EDTA, 0.2 M sucrose, pH 8, by gel filtration on Sephadex G-25 and applied to a 25-ml biuret column of DEAESephadex A-50 previously equilibrated with the same buffer. Elution was carried out with a linear NaCl gradient (0-0.4 M) in the same buffer to which 5 mM β-mercaptoethanol had been added. Aldolase B was present in the breakthrough peak with the hybrids B&Z, B&z, and BC following in that order, while aldolase C was eluted last. The fraction size was 1 ml.

previously employed for the resolution of the AC hybrid set (12). As shown in Fig. 1, aldolase B was not retained by the column under the conditions employed and was collected in the breakthrough peak. Aldolases B&z, B&z, BC, and C were eluted in that order from the column. The center fractions from each peak were used for further analysis.

The various members of the BC hybrid set were completely resolved from each other by this technique as shown in the cellulose-acetate electrophoresis activity stains presented in Fig. 2. The disc gel electrophoretic analyses in the lower part of the figure indicate that each of the heteromer preparations consisted of largely a single protein species with only minor detectable contaminants. The secondary bands seen in each of the gels may be caused by the presence of higher order aggregates of the aldolases since these species migrated in an ordered series behind those of the major bands. Such contaminants have been noted in ultracentrifugal studies of aldolase B (13).

RESULTS

Catalytic Properties of AC Aldolases—The reaction rates as a function of FDP concentration were determined for each member of the AC hybrid set isolated from rabbit brain. Fig. 3 presents data obtained for each of the hybrid species: A&z, A&z, and AC. A summary of the maximal velocities, Michaelis constants, and FDP:fructose-1-P ratios of the AC set are reported in Table I. The specific activities and FDP:fructose-1-P activity ratios of the heteromers fall in an ordered series between those of the parental homomers A and C while the Michaelis constants of all members of the set are very similar to each other. The values for each heteromer correspond closely to that predicted from the summation of the activities of the component subunits (in the appropriate proportions) of the heteromer.

These results can be explained by assuming statistical combination of subunits which exhibit the same specific catalytic characteristics in both heteromeric and homomeric configurations. However, the results can also be explained by a modulation of the activity of all subunits to form a tetramer of subunits with equivalent activities, each exhibiting the observed $V_{\text{max}}$.

The presence of identical or nonidentical catalytic units in an oligomer can be discriminated in systems in which considerable differences in the $K_m$ values of the component subunits are present. In this case, a molecule containing subunits with identical catalytic properties would produce typically linear reciprocal plots (neglecting secondary activations or inhibitions), while those containing subunits with nonidentical catalytic properties would yield curvilinear reciprocal plots whose shape should be predictable from the kinetic constants determined for the parent homomeric species (for a general discussion see Levitaki and Koshland (14)). Since aldolase B has considerably different catalytic properties than either A or C, a study of
FIG. 3. Catalytic activity as a function of FDP concentration: AC hybrids. The FDP cleavage activities of aldolases A, A, A, and A were determined with varying levels of FDP with the assay previously described (2) in 0.04 M Tris-Cl, pH 7.5, at 25°C. The data are plotted according to Woolf (see Haldane and Stern (15)) with [FDP] expressed in micromolar units.  

FIG. 4. Catalytic activity as a function of fructose-1-P concentration: BC hybrids. The fructose-1-P cleavage activities of aldolases B, B, B, and C were determined with varying levels of fructose-1-P as previously described (2). The data are plotted according to Woolf (15) with [fructose-1-P] expressed in millimolar units. ---, activities predicted for an equivalent mixture of the homomers B and C; 0, C; 0, B; 0, B.

### TABLE I

**Kinetic constants for aldolase AC hybrid set**

<table>
<thead>
<tr>
<th>Aldolase</th>
<th>( V_{\text{max}} ) (FDP cleavage)</th>
<th>FDP:fructose-1-P ratio</th>
<th>( K_m ) (FDP)</th>
<th>( K_m ) (fructose-1-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15-18</td>
<td>50</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>A,A</td>
<td>12-14</td>
<td>32</td>
<td>3</td>
<td>3</td>
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<td>32</td>
<td>3</td>
<td>3</td>
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<tr>
<td>A</td>
<td>5-8</td>
<td>10</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>5-8</td>
<td>10</td>
<td>2</td>
<td>4</td>
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### TABLE II

**Kinetic constants for members of aldolase BC hybrid set**

<table>
<thead>
<tr>
<th>Aldolase</th>
<th>( V_{\text{max}} ) (FDP cleavage)</th>
<th>FDP:fructose-1-P ratio</th>
<th>( K_m ) (FDP)</th>
<th>( K_m ) (fructose-1-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>B</td>
<td>2.1</td>
<td>0.9</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>B</td>
<td>3.2</td>
<td>0.9</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>B</td>
<td>4.0</td>
<td>0.9</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>C</td>
<td>5.2</td>
<td>0.9</td>
<td>1.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

### Notes:

- [fructose-1-P] = 0.01 M.
- [Tris-Cl] = 0.04 M.
- A test for effects of subunit interactions can be performed with fructose-1-phosphate since the B and C \( K_m \) values for this substrate (linear double reciprocal plots) differ by more than 10-fold. Reciprocal plots with fructose-1-phosphate were linear for the homomers B and C and were curvilinear for each of the heteromeric species. Fig. 4 presents specific results. Woolf plots, as discussed by Haldane and Stern (15), of the fructose-1-phosphate cleavage activities of the parental homomers B and C, and the heteromers B,C, and B,C are shown. The dashed lines represent theoretical plots of the molecules assuming the catalytic characteristics of B and C.

### Catalytic Properties of AC Aldolase Set—Table II presents the kinetic constants for members of the BC hybrid set. As with the AC heteromers, the \( V_{\text{max}} \) values for FDP cleavage are arranged in a series from B (1) to C (5). The \( K_m \) (FDP) values for the homomers B and C are relatively close and similar values were obtained for the heteromers. As expected, all of the double reciprocal plots were linear with FDP as a substrate. These data are similar to those obtained with the AC hybrid set and do not answer the question of identity or nonidentity of catalytic sites in heteromorphic combinations.

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**Catalytic Properties of BC Aldolase Set**—Table II presents the kinetic constants for members of the BC hybrid set. As with the AC heteromers, the \( V_{\text{max}} \) values for FDP cleavage are arranged in a series from B (1) to C (5). The \( K_m \) (FDP) values for the homomers B and C are relatively close and similar values were obtained for the heteromers. As expected, all of the double reciprocal plots were linear with FDP as a substrate. These data are similar to those obtained with the AC hybrid set and do not answer the question of identity or nonidentity of catalytic sites in heteromorphic combinations.

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combinative unit. This requires that each subunit contains at least one catalytically active site and, therefore, the tetramer must contain four or a multiple of four catalytically active sites.

**Immunochemical Properties of AC and BC Hybrid Sets**—The immunochemical properties of aldolase heteromers were examined with specific antisera prepared against purified aldolases $A_4$, $B_4$, and $C_4$. The results of double diffusion analyses of the AC hybrid set utilizing anti-aldolase A and anti-aldolase C sera are presented in Fig. 5. Anti-aldolase C reacted with $C_4$, $AC_3$, and $A_2C_3$ with an apparent reaction of identity and reacted with $A_4C_3$ to form a precipitin band with a spur. As indicated previously (12), there was no precipitation reaction of anti-aldolase C with aldolase A.

Conversely anti-aldolase A produced a reaction of identity with $A_4$, $A_2C$, and $A_2C_3$ while reacting very little if at all with $AC_3$ and not at all with $C_4$.

The spur information noted in the reaction of anti-aldolase C with $A_2C$ indicates that not all of the antibody molecules in the anti-aldolase C serum were capable of reacting with the isolated C subunit in $A_2C$. The lack of a visible reaction of anti-aldolase A with $AC_3$ may be caused by a similar phenomenon. Since the titer of the anti-A serum was lower than that of the anti-C serum, there may have been an insufficient concentration of such selected fractions of antibodies in the solution to form a visible precipitin band.

The results of the double diffusion analysis confirm earlier data indicating the specificity of the anti-aldolase sera. Antibodies react with the parental aldolase to which they are directed and do not cross-react with the other parental homomeric species. The present experiments show in addition that heteromers can react with either antibody. It is significant, however, that in heteromers containing an isolated subunit ($AC_3$ or $CA_3$) there was reaction with only a small fraction of the antibody population directed against that subunit. This fact might suggest an alteration in the structure of the isolated subunit upon combination in such tetramers, that a heteromer containing a single antibody site does not yield a precipitating complex, or that most antibodies are directed against determinants on more than one subunit.

To further examine the interactions of antisera with specific subunits, we took advantage of the ability of the antisera to specifically inhibit the catalytic activity of target aldolases (2). Mixing specific antisera with the appropriate homomer results in complete inhibition of FDP cleavage activity within 1 hour, even under low ionic strength conditions in which precipitation does not occur (2). If each specific antibody reacted quantitatively with its target subunit and if there were no significant subunit interactions, then the combinative proportions of each subunit type in the heteromer species could be titrated by the antisera.

**Effects of specific antisera on the catalytic activity of members of the AC and BC hybrid sets** were measured. The results are reported in Table III. As previously reported (2) the activity of the homomers was essentially completely inhibited by their specific antisera. Surprisingly, the FDP cleavage activities of all heteromeric molecules were also marked inhibited by antibodies specific for either kind of subunit; the inhibitions bore little or no relationship to the combinative proportions within the tetramer. Complete inhibition of activity of a heteromer was produced by an antibody when its target subunit was present in 2 or 3 positions in the tetramer, and more than 50% inhibition was observed when the target subunit was present as only one of the four subunits in the molecule. The values recorded in Table III were obtained with high anti-aldolase A and anti-aldolase C sera. The inhibitions expressed are relative to control incubations of the same proteins with normal chicken serum.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$A_4$</th>
<th>$A_2C$</th>
<th>$A_2C_3$</th>
<th>$AC_3$</th>
<th>$C_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-C</td>
<td>90</td>
<td>95</td>
<td>%</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Anti-A</td>
<td>100</td>
<td>95</td>
<td>97</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Anti-B</td>
<td>88</td>
<td>96</td>
<td>99</td>
<td>95</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table III**

**Inhibition of catalytic activity of AC and BC hybrid sets by specific antibodies**

The various aldolases were incubated with antibody as previously described (2). The inhibitions expressed are relative to control incubations of the same proteins with normal chicken serum.

Fig. 5. Double diffusion analysis: AC hybrid set. A double diffusion analysis of various members of the AC hybrid set was performed as described previously (2) with antibodies prepared against aldolases A and C. Left, anti-aldolase A was placed in the center wells of both plates. Top: 1, aldolase $A_4$, 1.0 mg per ml; 2, aldolase $A_2C$, 1.0 mg per ml; 3, aldolase $A_2C_3$, 1.0 mg per ml. Bottom: 1, aldolase $A_4$, 0.25 mg per ml; 2, aldolase $A_2C$, 0.33 mg per ml; 3, aldolase $A_2C_3$, 0.50 mg per ml; 4, aldolase $AC_3$, 1.0 mg per ml; 5, aldolase $C_4$, 1.0 mg per ml; 6, aldolase $AC_3$, 1.0 mg per ml. Right, anti-aldolase C was placed in the center wells of both plates. Top: 1, aldolase $A_4$, 1.0 mg per ml; 2, aldolase $A_2C$, 1.0 mg per ml; 3, aldolase $A_2C_3$, 1.0 mg per ml; 4, aldolase $AC_3$, 1.0 mg per ml; 5, aldolase $A_4$, 1.0 mg per ml; 6, aldolase $A_2C$, 1.0 mg per ml; 7, aldolase $A_2C_3$, 1.0 mg per ml; 8, aldolase $AC_3$, 1.0 mg per ml; 9, aldolase $C_4$, 1.0 mg per ml; 10, aldolase $AC_3$, 1.0 mg per ml.
were incubated with a constant amount (25 μl) of anti-aldolase C for 1 hour at 4°C in a nonprecipitating system described previously (2). FDP cleavage assays were performed at 25°C (2). ■, C4; ○, AC2; ▲, AxC,C7; O, AxC.

antiserum alone (~ 0.02 μmole of FDP cleaved per min per ml of antiserum). The values thus represent minimal estimates of the theoretical inhibitions. Fig. 6 represents a titration of anti-C serum by the various members of the AC hybrid set. C4, the original antigen, is recognized by the highest proportion of antibodies (or is a better antigen), AC5 the next highest, and so on. The total cross specificities of the apparently diverse antibody populations must await studies on the various antibody species purified by specific absorption.

**Discussion**

A number of earlier studies have shown that the subunits of aldolases A, B, and C can interact to form stable hybrid tetramers (9). In the present studies, these heteromers have been isolated and the properties of both homomeric and heteromeric combinations of subunits have been examined. The results bear on the following points (a) interactions of subunits in oligomer formation; (b) the number of active sites in the aldolase tetramer; (c) the physiological function of the heteromers; and (d) the interaction of antibodies with specific proteins.

The kinetic experiments indicate that there is little change in the measured catalytic properties of subunits whether in homomeric or heteromeric combination. The graded series of \( V_{\max} \), specificity (FDP:fructose-1-P activity ratio), and the \( K_m \) values of all members of the hybrid sets were within the limits of measurement of those predicted from the subunit compositions assuming that each subunit in a homomeric species behaves identically and that there is no influence of heteromeric combination on catalytic behavior. Whereas other explanations can be given to the \( V_{\max} \) and FDP:fructose-1-P activity ratios, there is no other plausible interpretation for the precise agreement of the curvilinear Woolf plots obtained for the fructose-1-P cleavage reactions catalyzed by the BC heteromers with those expected from the proposed interpretation. The possibility that these data may be explained by a cooperative effect of substrate on the reaction rates of the BC heteromers when none is found with fructose-1-P on either of the homomers or the AC heteromers or with FDP in any aldolase system tested seems very remote and is, therefore, discounted. We, therefore, conclude that homomeric or heteromeric interaction of the subunits has no (or the same) effect on their catalytic behavior. This conclusion has physiological, catalytic, and structural consequences.

From a physiological point of view, a cell producing two aldolase subunit types is assured that the specific characteristics of those subunits will be expressed whether they exist in homomeric or heteromeric combinations. Thus, regulation of the production of subunits in all cells can occur independently at the two genetic loci in response to an appropriate metabolic need. If the heteromers exhibited unique physiologically significant properties, then appropriate regulation of the production of the two subunits might be independent in tissues in which only one subunit was present, but coupled in tissues in which both subunits were found. Dual regulation of this sort seems cumbersome both in its metabolic and in its genetic aspects; it is apparently not involved in the case of the aldolases.

From a catalytic point of view, strict correspondence between the activity of heteromers with the distribution of subunit types requires that each subunit has an equivalent number of catalytically active sites. Thus, aldolase tetramers must have at least four statistically equivalent active sites. This significant conclusion is at variance with the results of a number of previous investigations indicating three active sites per molecule (9-11). Although no previous study of any FDP aldolase has found four active sites, we consider the present evidence to be compelling. The failure of direct binding studies to obtain this stoichiometry (10) is most likely caused by the inability of all subunits in the preparation to react with the substrate or substrate analogues employed. Some of the subunits may be nonfunctional (because of inactivation during isolation, or even within the cell). Whatever the reason, the difficulties in obtaining a reliable view of over-all structure-function relationships in enzyme molecules from simple stoichiometric determinations which inherently yield minimal values are re-emphasized.

From the structural point of view, the conclusion of independent catalytic expression of the different combinatorial units in a heteromer gives no indication of functional interaction between the subunits in these molecules. Such interactions may, however, exist without affecting the properties of the catalytic sites. The inhibition of the catalytic activity of both types of subunits in a heteromer by specific antibodies prepared against either subunit provides suggestive evidence for significant interactions of the subunits within the tetramer. There are several possible explanations for this phenomenon. (a) Reaction of antibody with a single subunit in a tetrameric molecule results in substantial inhibition of the entire molecule via conformational changes in several if not all of the subunits. (b) There may be an interaction between subunits in both heteromers and homomers with the consequence that new immunologically active sites are uncovered in the subunits of the heteromer. Thus, whereas the three major classes of subunits A, B, and C do not cross-react immunologically in homomeric configurations, they may cross-react in heteromeric configurations. Thus, the environment may progressively influence the structure of a particular subunit within the tetramer. (c) Finally, reaction of the antibody with aldolases might cause dissociation of the molecules into inactive subunits which are prevented from reassociating somehow in the system employed. Any of the
possible explanations imply a functionally significant interaction between subunits.

A more detailed investigation of the molecular basis of the interactions of antibodies with aldolase heteromers and homomers may clarify significant features of the nature of aldolase subunit interactions and perhaps in addition some characteristics of the antigen-antibody reaction.

In a more general context, the present experiments emphasize the utility of employing homomeric and heteromeric molecular families to obtain relevant information concerning structure-function relationships in oligomeric molecules. Certainly a more complete physical, chemical, and catalytic study of the aldolase hybrid sets will provide an insight into the basic characteristics of the molecules and the basic rules governing subunit chemistry. This approach may be used to advantage in other systems as well. For example, if it were possible to "freeze" the subunits of an allosteric protein (i.e. by covalent interaction with an effector), then dissociation, formation of hybrids, subsequent resolution and analysis, might allow an unambiguous elucidation of the molecular basis for certain types of regulatory phenomena.

Acknowledgments—We wish to acknowledge the valuable collaboration of Dr. Walter Susor in the analysis of the catalytic properties of the aldolase BC hybrid set.

REFERENCES
Catalytic and Immunochemical Properties of Homomeric and Heteromeric Combinations of Aldolase Subunits
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