*NONIDENTITY OF SUBUNITS OF RABBIT MUSCLE ALDOLASE**

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Although rabbit muscle aldolase is known to be composed of polypeptide subunits, there is considerable uncertainty as to the number and nature of these subunits. The enzyme has been shown to contain three carboxy-terminal tyrosine residues[1] and to dissociate in urea,[2] in acid,[2, 3] or at alkaline pH[4] into chains having one third the molecular weight of the native enzyme. A model for the enzyme structure based on three similar subunits is supported by the demonstration that the native enzyme contains three substrate-binding sites.[5–7] On the other hand, the enzyme has been reported to dissociate in guanidine hydrochloride into four approximately equal subunits,[8] and a model based on four similar subunits is supported by recent experiments based on hybridization of the subunits of muscle, liver, and brain aldolase.[9]

The nonidentity of the subunits in aldolase was originally suggested by the observation that prolonged digestion with small amounts of carboxypeptidase releases three carboxy-terminal tyrosine residues, but only two of the three penultimate alanine residues.[10, 11] Support for a three-chain AAB model was derived from further experiments with carboxypeptidase,[12] from disk gel electrophoresis in 8 *M* urea,[13] and from the number of peptides formed on digestion with trypsin or following degradation with cyanogen bromide.[13]

We have now obtained direct evidence for the presence of two distinct subunits in rabbit muscle aldolase which can be separated by DEAE-cellulose chromatography in 8 *M* urea. The two chains differ significantly in amino acid composition and in peptide fingerprint patterns. They are similar in molecular weight and each carries the Schiff base-forming site. In the present publication we report the separation and characterization of these subunits.

Materials and Methods.—Fructose diphosphate aldolase (FDP aldolase) was prepared from rabbit muscle by the method of Taylor[14] and recrystallized several times. Aldolase activity was assayed spectrophotometrically, as described by Racker,[15] and the unit of activity defined as the amount required to cleave 1 μmole of FDP per minute. Protein concentration was determined by measurement of absorbancy at 280 mμ and calculated using the value 0.91 for the absorbance of a solution containing 1 mg of protein per ml.[16] β-Glycerophosphate aldolase was prepared as described by Lai *et al.*[17] The protein was carboxymethylated at concentrations of 1–5 mg per ml, according to Crestfield *et al.*,[18] except that the reaction was carried out at pH 7.5 instead of pH 8.6. Native aldolase was carboxymethylated in the same way.

Uniformly labeled C^{14}-dihydroxyacetone phosphate (DHAP) was prepared by modification of the method previously described,[19] and was purified by high-voltage electrophoresis on paper. DEAE-cellulose was purchased from Reeve Angel and Co. and washed successively with 0.5 *N* HCl and 0.5 *N* NaOH before it was equilibrated with 0.1 *M* Tris-HCl buffer, pH 7.5. Fine particles were removed by repeated decantation. Urea (certified A.C.S. grade) was obtained from the Fisher Scientific Company and either recrystallized from 95% ethanol or used immediately after deionizing the solutions on Amberlite MB3 resin columns. Fractions from the chromatographic columns were analyzed for protein by their absorbancy at 280 mμ. Radioactivity of samples in urea solution were determined with aliquots dried on filter paper strips which were placed in vials containing 15 ml of a phosphor in toluene.[20] Fractions from the columns were dialyzed immediately against water and lyophilized.

Disk gel electrophoresis was carried out with a Canalco model 12 apparatus, using the pH 8.5 polyacrylamide gel system of Ornstein and Davis[21] and the pH 4.3 system of Reisfeld *et al.*[22] The acrylamide monomer concentration was 7.5%. Gels in 8 *M* urea were prepared by adding the required amount of freshly recrystallized urea to the various solutions immediately before polymerization. The increase in volume resulting from the addition of urea reduced the monomer concentration to 4.5%. No urea was added to the electrolyte buffer in the reservoirs, but mercaptoethanol (14 mM) was added when protein samples had not been carboxymethylated. Electrophoresis was carried out for $1^1/_2$ hr at a constant voltage which varied in individual experiments from 200 to 300 volts, and an initial current of 5 ma per tube. The protein bands were stained with amido blue-black. For a comparison of band intensities, photographic transparencies were taken and densitometric measurements were made using a Joyce double-beam recording microdensitometer Mk IIIc.

Sedimentation velocity measurements were carried out at ambient temperature, with a Spinco model E ultracentrifuge run at 59,780 rpm using schlieren optics. The samples contained 1.5–5 mg of protein per ml in 2% acetic acid containing 0.05 *M* NaCl. The results were corrected for the solvent viscosity and for the effect of temperature on the viscosity of water and on the density of the solvent. The results reported were derived by extrapolation to zero protein concentration. The molecular weight of the subunits was also estimated by gel filtration of Sephadex G-100, as described by Andrews.[23] Each sample contained Blue Dextran (mol wt = 2×10^6) as an internal standard and was eluted with 8 *M* urea in 0.1 *M* Tris-HCl buffer containing 0.14 *M* mercaptoethanol and 1 mM EDTA. Proteins used as standards were egg-white lysozyme (recrystallized, mol wt = 15,000), *E. coli* alkaline phosphatase (purified, monomer mol wt = 40,000), and bovine serum albumin (recrystallized, mol wt = 69,000).

Amino acid analyses were carried out on samples hydrolyzed in 6 *N* HCl under vacuum for 22 hr at 110°. The hydrolysates were evaporated to remove HCl and taken up in 0.2 *M* Na citrate buffer, pH 2.4. Analyses were carried out with a Spinco model 120B automatic amino acid analyzer adapted for high-sensitivity determinations. Reproducibility of the results under these conditions was found to be within ±2%. Tryptophan was determined by measurement of absorbancy at 280 mμ at pH 2.8, corrected for the tyrosine content as determined from the amino acid analyses.

Cleavage with cyanogen bromide was carried out as described by Steers *et al.*[24] The protein samples were dissolved in 70% formic acid at a concentration of 1 mg per ml and treated with a 50-fold excess (w/w) of cyanogen bromide (0.4 g per ml) in 70% formic acid. The mixtures were kept for 20 hr at 25°, diluted 10 times, and lyophilized. For the preparation of proteolytic digests, trypsin treated with *L*-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) according to the method of Kostka and Carpenter[25] was employed. The protein samples (2.9 mg) were suspended in 0.1 *M* ammonium bicarbonate, pH 8.6, at 37°, and 30 μg of TPCK-trypsin was added in three equal aliquots over a period of 3 hr. Proteolysis was found to be complete within 4 hr, after which the samples were lyophilized. Aliquots of the digested samples, corresponding to 0.5 mg of protein, were placed on Whatman 3MM filter paper sheets in a 1-cm streak. Electrophoresis was carried out[26] with the filter paper samples placed between two thin pyrex glass sheets, resting on a horizontal water-cooled aluminum plate which had been covered with a thin layer of glycerol. Efficiency of heat transfer was further ensured, and diffusion of buffer into the filter paper reduced, by placing heavy glass plates on top of the upper pyrex sheet. Electrophoresis was performed at 30 v/cm for 3 hr, with a buffer of pyridine:acetic acid:water (10:1:89) at pH 6.0. Samples to be compared were run simultaneously. After electrophoresis the two papers were dried in air overnight and then subjected to ascending chromatography in the second dimension in a large tank at 20°; the solvent system was pyridine:isoamyl alcohol:water (35:35:30).[27] When the solvent had risen to 17 cm, the papers were dried in air and, finally, in a 90° oven for 5 min.

Peptides were located by dipping the papers in a 0.3% (w/v) solution of ninhydrin and acetone, followed by heating in a 90° oven for 5–15 min. Peptides containing arginine were then located by the modified Sakaguchi technique developed by Irreverre.[28] Radioactive spots were located by autoradiography for 4–7 days using Kodak "No-Screen" X-ray film.

Results.—Evidence for the existence of nonidentical subunits: Nonidentical subunits were first detected in disk gel electrophoresis in 8 *M* urea at pH 8.5. Native

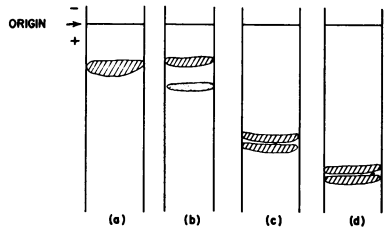

FIG. 1.—Disk gel electrophoresis patterns at pH 8.5 (see *Methods*). The patterns were obtained by tracing projected images of photographs of the gels. (*a*) Native aldolase, (*b*) aldolase in 8 *M* urea, (*c*) carboxymethyl aldolase, and (*d*) CM-β-glycerophosphate aldolase.

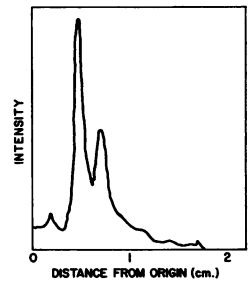

FIG. 2.—Densitometric tracing of the pattern obtained in disk gel electrophoresis of aldolase in 8 *M* urea.

aldolase migrated as a single protein band (Fig. 1*a*). In 8 *M* urea the same sample was found to yield two well-separated bands (Fig. 1*b*). The pattern obtained in the presence of urea was analyzed by densitometric measurement (Fig. 2). The areas under the two peaks, corresponding to the intensities of the bands, were found to be in the ratio of approximately 2:1.

In disk gel electrophoresis in urea, S-carboxymethyl aldolase (CM-aldolase) also yielded two bands which migrated considerably more rapidly than those obtained from native aldolase under the same conditions (Fig. 1*c*). This reflects the increased electrophoretic mobility due to the negative charges of the additional carboxymethyl groups. Carboxymethylation was shown to be complete by employing C^{14}-labeled iodoacetate. In two experiments, 27.3 and 28.3 carboxymethyl groups were incorporated per mole of enzyme, as expected for complete alkylation of the cysteine residues.[29]

Disk gel electrophoresis of β-glycerophosphate aldolase: The proteins in both bands were shown to contain the sites capable of forming the Schiff base intermediate with the substrate. These were labeled by treating the native enzyme with borohydride in the presence of DHAP.[17] The enzyme, which was 97 per cent inactivated by this treatment, was carboxymethylated as described above and analyzed by disk gel electrophoresis. The labeled preparation yielded two bands similar to those of CM-aldolase, but which migrated more rapidly (Fig. 1*d*). The increase in electrophoretic mobility was found to be approximately the same for each band, suggesting that each contained the additional negative charges introduced by the β-glycerophosphate residue. This was confirmed by labeling the active sites with C^{14}-DHAP and measuring the radioactivity in each band after electrophoresis. Both bands were found to contain radioactivity (see below).

Separation of the subunits by column chromatography: The two types of subunits were separated by chromatography of CM-aldolase on DEAE-cellulose columns in 8 *M* urea. In these experiments, three major protein peaks were obtained (Fig. 3). These were examined by disk gel electrophoresis in 8 *M* urea (Fig. 4). The material from peaks *A* and *B* of the DEAE chromatogram each yielded a single band in disk gel electrophoresis in urea at pH 8.5, corresponding to the upper and lower bands, respectively, of unfractionated CM-aldolase. Peak *C* was a hetero-

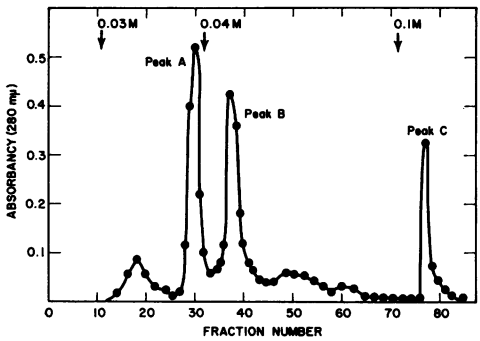

FIG. 3.—Chromatography of CM-aldolase. A DEAE-cellulose column (1 × 30 cm) was equilibrated in 5 mM Tris-HCl buffer, pH 7.5, containing 8 *M* urea (starting buffer). The sample of CM-aldolase (26.3 mg dissolved in 5.0 ml of starting buffer) was placed on the column and eluted by stepwise increases in the molarity of Tris-HCl. The arrows indicate the buffer changes following elution with 50 ml of starting buffer; all buffers contained 8 *M* urea. The flow rate was 12 ml per hr and fractions of 4 ml were collected.

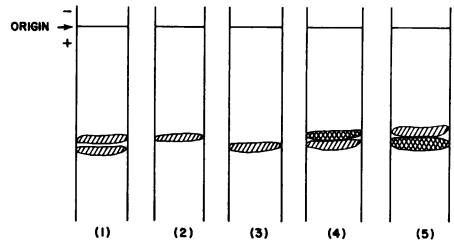

FIG. 4.—Analysis by disk gel electrophoresis of fractions obtained in DEAE-cellulose chromatography of CM-aldolase. (*1*) Unfractionated CM-aldolase, (*2*) α subunit (peak *A*), (*3*) β subunit (peak *B*), (*4*) α subunit added to unfractionated CM-aldolase, and (*5*) β subunit added to unfractionated CM-aldolase.

geneous mixture, containing neither of the bands present in the original material, and was apparently composed of artifacts formed during prolonged exposure to urea. When fractions A and B were added to the starting material, the patterns obtained showed the expected intensification of the corresponding bands (Fig. 4).

In this experiment, the total recovery of protein from peaks *A* and *B* was 49.1 per cent. Because of the low yield, it was not possible to estimate the proportions of A and B in the original enzyme.

Properties of the separated subunits: (1) *Sedimentation velocity:* The separated subunits were analyzed by sedimentation velocity centrifugation in 2 per cent acetic acid and the results, extrapolated to infinite dilution, yielded values of $s^0_{20,w}$ (±5%) of 1.9 and 2.0 for the α subunit (peak *A*) and β subunit (peak *B*), respectively. Unfractionated CM-aldolase yielded a value of 2.0 under these conditions. In each case, the schlieren pattern showed a single symmetrical peak. The $s^0_{20,w}$ values are to be compared with the value of 2.0 for aldolase subunits dissociated by urea, as reported by Stellwagen and Schachman.[2] The subunits isolated by chromatography thus appear to be similar in size to the subunits which have been observed in dissociation media by other workers.[2, 3]

(2) *Estimation of molecular weight of the isolated subunits by gel filtration:* Sedimentation equilibrium measurements were found to be unsuitable for the determination of the molecular weight of the subunits, because they formed aggregates at neutral pH, and were found to undergo degradation with the formation of heterogeneous mixtures on prolonged exposure to acid pH (pH 2–3). We therefore compared the isolated carboxymethylated subunits with aldolase (not carboxymethylated) in 8 *M* urea by gel filtration on Sephadex G-100. Each sample was found to emerge as a single protein peak; the elution volumes of the isolated carboxymethyl subunits and those derived from unmodified aldolase were identical, and corre-

TABLE 1

AMINO ACID COMPOSITION OF α AND β SUBUNITS

Amino acid	α Subunit	β Subunit	Amino acid	α Subunit	β Subunit
Lys	30.2	34.3	Ala	58.3	55.2
His	14.0	15.4	Val	24.5	22.4
Arg	18.8	22.5	Met	2.8	3.2
CM-Cys	6.5	9.5	Ileu	22.7	22.1
Asp	38.4	37.5	Leu	44.3	44.3
Thr	29.9	26.7	Tyr	14.6	15.9
Ser	27.0	25.3	Phe	9.2	9.1
Glu	55.4	51.6	Try	4.0	4.5
Pro	23.7	21.9	Total	465.5	459.2
Gly	41.2	37.8			

Twenty-two-hour hydrolysates of the α and β subunits, each containing 0.125 mg, were analyzed as described in *Methods*. The results are expressed as moles of amino acid per mole of subunit, assuming a molecular weight of 50,000 for each subunit.

sponded to a molecular weight of 52,000 ± 6,000. The purified subunits were indistinguishable in size from those formed by dissociation of aldolase in urea.

(3) *Amino acid composition:* The two subunits were similar in amino acid composition (Table 1), although there were significant differences in the content of lysine, arginine, threonine, and cysteine.

(4) *Tryptic peptide patterns:* Consistent with these differences in amino acid composition were the differences in peptide patterns obtained in two-dimensional fingerprints of the α and β subunits (Fig. 5). The total number of ninhydrin-positive spots which could be detected in the β subunit was five more than in the α subunit; on the basis of the difference in lysine and arginine content, eight additional peptides could have been formed. The β subunit, which contains four extra arginine residues, yielded three additional arginine-containing peptides. This subunit also yielded three additional radioactive spots in experiments with C^{14}-carboxymethyl aldolase, consistent with the presence of three extra cysteine residues. As seen in Figure 5, each subunit yields a few unique tryptic peptides which are absent in the digests of the other subunits. The fingerprint pattern obtained from unfractionated CM-aldolase was as expected for a mixture of the α and β subunits.

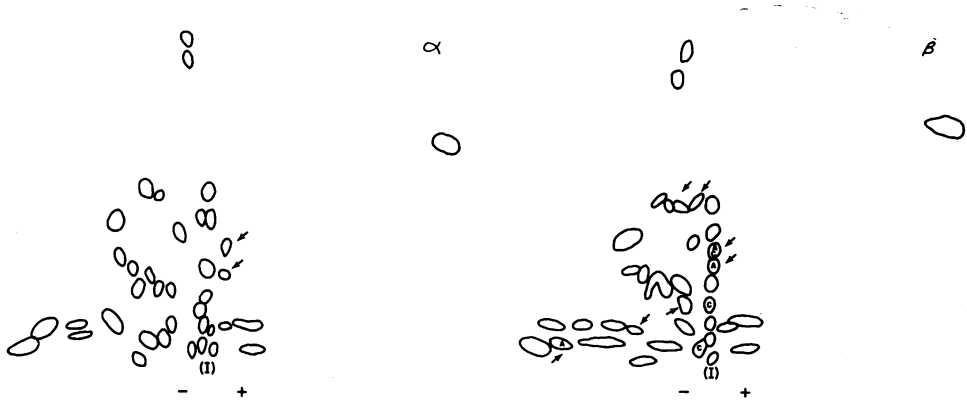

FIG. 5.—Fingerprint patterns of 0.5 mg of tryptic digest of samples of each of the carboxymethylated subunits α and β. The outlines indicate the positions of ninhydrin-positive spots. The unique peptides in each subunit are indicated by arrows. Extra arginine or CM-cysteine spots are indicated by *A* and *C*, respectively. Electrophoresis and chromatography are shown in the horizontal and vertical dimensions, respectively.

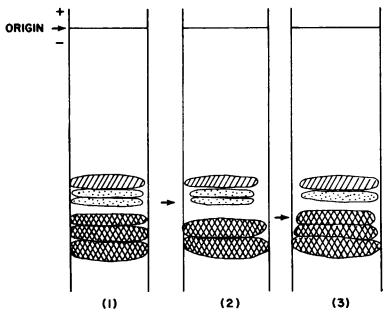

FIG. 6.—Disk gel electrophoresis patterns of cyanogen bromide fragments from unfractionated CM-aldolase (*1*), the α subunit (*2*), and the β subunit (*3*). The arrows indicate the unique peptides.

(5) *Patterns obtained after cleavage with cyanogen bromide:* Since aldolase contains relatively few methionine residues, cleavage with CNBr was expected to yield a small number of peptides which might show differences between the subunits.[13] CM-aldolase and the isolated carboxymethyl subunits α and β were treated with CNBr and the cleavage products examined in disk gel electrophoresis in 8 *M* urea at pH 4.3. CM-aldolase yielded six major bands, four of which were common to both subunits, with each subunit also yielding a single unique band (Fig. 6). From these results, the subunits appear to contain large regions of similar primary structure with other regions showing distinct differences.

(6) *The COOH-terminal amino acids:* During digestion with carboxypeptidase A, both subunits released 1.0 equivalent of tyrosine per 50,000 molecular weight, followed by 1.0 equivalent of alanine, consistent with results reported previously with the intact protein.[12] This proves that the α and β subunits are not simply the result of COOH-terminal degradation. Further digestion with carboxypeptidase indicated significant differences in the COOH-terminal peptides, similar to those previously reported by Winstead and Wold.[12] These results will be presented in detail elsewhere.

(7) *Evidence for the presence of an active site in each subunit:* As indicated earlier, CM-β-glycerophosphate aldolase yielded two bands in disk gel electrophoresis (Fig. 1*d*). Preparations which had been partially converted to the β-glycerophosphate derivative and then carboxymethylated showed four bands, two corresponding to the subunits of CM-aldolase and two to the subunits of CM-β-glycerophosphate aldolase. Such a sample, containing 78 per cent of CM-β-glycerophosphate aldolase labeled by reduction in the presence of C^{14}-DHAP, was fractionated on DEAE-cellulose in 8 *M* urea as described above, and the fractions were analyzed by disk gel electrophoresis. The unlabeled α and β subunits were eluted with the 0.03 *M* Tris-HCl buffer; these were not radioactive (Table 2). Two additional peaks were eluted with 0.035 *M* and 0.04 *M* Tris-HCl buffer, respectively. Each was radioactive and was found to contain nearly one equivalent of C^{14}-β-glycerophosphate per 50,000 molecular weight. On disk gel electrophoresis peaks 3 and 4 yielded

TABLE 2

CHROMATOGRAPHY OF PARTIALLY LABELED CM-C^{14}-GLYCEROPHOSPHATE ALDOLASE

	Total protein recovered* (mg)	Radioactivity		Incorporation of C^{14}-glycerophosphate† (equiv/subunit)
		Total (cpm)	Specific (cpm/mg)	
Peak 1	3.5	0	0	0
Peak 2	1.4	0	0	0
Peak 3	8.2	47,500	5,800	0.83
Peak 4	8.0	45,900	5,700	0.81

* The sample (48 mg) was placed on a DEAE-cellulose column (1.5 × 35 cm) and eluted as described in the text (see also Fig. 3). The specific radioactivity of the sample placed on the column was 4700 cpm/mg.

† The specific activity of the C^{14}-dihydroxyacetone phosphate was 3.52 10^6 cpm/μmole.

single bands corresponding to the upper and lower bands, respectively, obtained with CM-β-glycerophosphate aldolase. The specific radioactivity of the purified subunits was found to be 5700–5800 cpm per mg. Using this value, we calculated that the unfractionated material (specific activity = 4700 cpm per mg) consisted of 81 per cent labeled subunits and 19 per cent unlabeled subunits. This is in close agreement with the estimate based on loss of catalytic activity after reduction with borohydride, which yielded values of 78 per cent labeled protein and 22 per cent unlabeled protein.

Following elution of peak 4, a large peak containing the remainder of the protein was eluted with 0.1 *M* Tris-HCl buffer, as was found in the experiment with CM-aldolase (see Fig. 3). On disk gel electrophoresis this yielded a large number of bands, none of which corresponded to any in the original preparation. These were obviously artifacts produced during the long exposure to 8 *M* urea.

Homogeneity of the crystalline aldolase preparations: While the results described here support the conclusion that aldolase is composed of nonidentical subunits, they do not exclude the possibility of a mixture of two isozymes. We have not been able to obtain any indication of the presence of isozymes in our preparations of muscle aldolase in chromatographic and electrophoretic experiments. The twice-recrystallized enzyme (60 mg) was chromatographed on a column (1.5 × 38 cm) of DEAE-cellulose at pH 8.4, and was found to elute as a single symmetrical peak containing 96 per cent of the applied activity and protein. In polyacrylamide disk gel electrophoresis at pH 8.5 (Fig. 1*a*) and at pH 4.3 (not shown), the enzyme migrated as a single protein band. Under no conditions was heterogeneity detected.

Attempts to obtain hybrid forms from the muscle aldolase were unsuccessful. When the enzyme was dissociated at pH 2.0 and allowed to reassociate at pH 7.5, as described by Penhoet *et al.*,[9] 95 per cent of the original enzymatic activity was regained. Electrophoretic examination of the reconstituted enzyme at pH 4.3 showed the presence of a single species indistinguishable from the native enzyme, in agreement with the results of Penhoet *et al.*[9] We may thus conclude that the subunits which have been described did not originate from isozymes of rabbit aldolase, and that the manner in which the dissociated nonidentical subunits may recombine is subject to significant restrictions.

Discussion.—On the basis of the results reported here, it may be concluded that rabbit muscle aldolase contains two different types of subunits. Support for a three-chain (AAB) model is provided by the experiments which demonstrate that all of the subunits contain an active site, since no unlabeled subunit remained in the enzyme after complete inactivation in the presence of DHAP and borohydride. Several independent reports indicate that the number of active sites is three.[5–7] Thus, our data, which exclude the presence of a fourth unlabeled subunit, are consistent with the presence of only three subunits. Further support for this suggestion is provided by the relative intensities (2:1) of the bands obtained in polyacrylamide gel electrophoresis of the enzyme in 8 *M* urea.

The two types of subunits appear to be similar, but not identical, in their primary sequence. Peptide patterns from the two subunits, obtained after tryptic digestion or cleavage with CNBr, showed over-all similarities, although with significant differences. Both subunits were found to contain the lysine residue which forms the Schiff base intermediate with the substrate, dihydroxyacetone phosphate. Pre-

vious work on the isolation and analysis of an active site peptide from rabbit muscle aldolase[30] does not permit a definite conclusion regarding the presence or absence of different primary sequences around the reactive lysine residues.

The presence of two nonidentical peptides in rabbit muscle aldolase raises a number of questions regarding the interpretation of the hybridization experiments recently reported by Penhoet *et al.*[9] The number of species formed in the random hybridization of enzymes containing nonidentical subunits would be considerably greater than the number which was detected in their hybridization experiments. This might be due to the formation of hybrids having similar or identical electrophoretic mobilities, which would not be detected by the methods employed. Our finding that a mixture of nonidentical subunits reassociates to yield a single molecular species (muscle aldolase) and does not generate new hybrid combinations indicates that there exists a preferential or necessary mode of recombination (AAB) between the different subunits. Such restrictions might explain the results obtained in the hybridization experiments.

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† Predoctoral trainee of the National Institutes of Health (GM 1191).

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