An Investigation of the Subunit Structure of Yeast Enolase*

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ABSTRACT: Yeast enolase was digested with either cyanogen bromide or trypsin. The peptides were examined to see if the subunits of the enzyme are identical. On disc electrophoresis in 4 M urea at pH 2.3, four major cyanogen bromide peptides were found. Analysis of the amino terminals of the peptides showed only alanine and four to five times as much glycine. On peptide mapping of tryptic digests of enolase, 46 ± 2 peptides were obtained, 3 of which stain positively for tryptophan, 8–12 for arginine, 6 for histidine, and 7 for tyrosine. From the amino acid composition of the enzyme, it is concluded that these facts are more consistent with two identical subunits than two different ones. This conclusion is supported by the fact that we detected 1.76 ± 0.19 moles of amino-terminal alanine/mole of enolase, and by the fact that the amino-terminal sequence is Ala-Gly-Lys-Val-Gly-Asp-Thr-Glu(NH_2) for both subunits.

Malmström *et al.* (1959) determined the amino acid composition and carboxyl and amino terminals of yeast enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11). They found 0.9 mole (corrected) of N-terminal alanine and an unquantitated amount of C-terminal leucine. They concluded that yeast enolase was a single polypeptide chain of 67,000 molecular weight. Later, Brewer and Weber (1968) showed that the enzyme could be dissociated into two subunits of approximately equal molecular weight, a finding which was confirmed by Gawronski and Westhead (1968). These results were reconciled to those of Malmström *et al.* (1959) by suggesting that the two subunits were of different primary sequence (Brewer and Weber, 1968).

Brewer and Weber (1966) had earlier shown that 1 mole of tightly bound magnesium produces significant structural changes in the enzyme, and Hanlon and Westhead (1969a,b) have recently presented evidence suggesting that a second, less strongly bound magnesium ion is more involved in catalysis. In order to further define the function(s) of magnesium in activating this enzyme, it is necessary to see whether the two subunits are identical in sequence or not, since very different mechanisms of activation can be entertained in the two cases. To this end, we have examined the primary structure of yeast enolase by peptide mapping and by quantitative analysis of the N-terminal amino acid sequence.

Materials and Methods

Yeast enolase was prepared by the method of Westhead and McLain (1964). Separation of the mixture of enolases A, B, etc., was achieved by chromatography on triethylaminoethylcellulose (Figure 1) by a modification of the method of Westhead and McLain (1964). Enolase A, which is the native form according to Westhead and McLain (1964), and B were used in this paper. Enolase A was estimated to be at least 95% homogeneous by disc electrophoresis at several pH values (Figure 2); enolase B was usually contaminated with some enolase A. For this reason, enolase A was used in peptide mapping experiments. The various forms of enolase are thought to differ only in the number of amide groups, since they have the same specific activity, Michaelis constants, molecular weight, amino acid composition, and N terminus (Westhead and McLain, 1964). The specific activities of enolases A and B prepared by us were about twice the values reported by Westhead and McLain (1964), which in turn were about 20% higher than that of the material used by Malmström *et al.* (1959).

N-Ethylmorpholine, acrylamide, glycine, *N,N'*-methylenebisacrylamide, Tris, trifluoroacetic acid, tetrahydrofuran, methyl isothiocyanate, and *N,N,N',N'*-tetramethylethylenediamine were obtained from Eastman Kodak and redistilled, recrystallized, or otherwise purified as required (Brewer and Weber, 1966; Richards *et al.*, 1969; Brewer and Ashworth, 1969). Other reagents were analytical reagent grade from several sources, notably Matheson, Coleman & Bell and the Baker Chemical Co. Carboxypeptidase B was purchased from Worthington, and triethylaminoethylcellulose from Bio-Rad.

Cyanogen bromide digestion of enolase was carried out for 1–6 days, in 70% formic acid at room temperature (Gross, 1967). Digestion for different times had no effect on the disc electrophoretic patterns produced by the peptide mixtures. A 30–3000-fold excess (over the methionine content of the enzyme) of cyanogen bromide was added. Following digestion, the sample was lyophilized four times with addition of water between dryings. The cyanogen bromide peptides were dissolved in water or 8 M urea before analysis.

For tryptic digestion, the enzyme was treated with 0.01 M EDTA, and this and other salts were removed by dialysis in plastic beakers against several changes of doubly distilled water. The protein was lyophilized and dissolved in 0.2 M *N*-ethylmorpholine acetate (pH 8.5). For digestion, porcine trypsin was used (Travis and Liener, 1965). The trypsin had previously been checked for chymotrypsin-like esterase activity. None was found. However, as a precaution, the trypsin was treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Carpenter, 1967) to eliminate any possible traces of chymotryptic activity. The trypsin was

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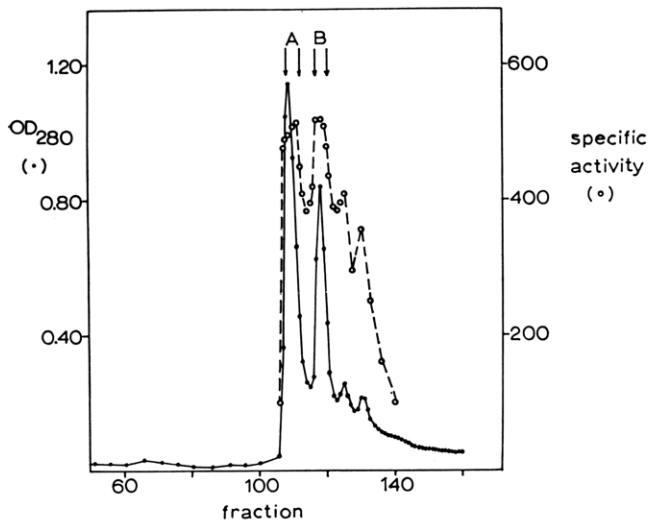

FIGURE 1: Isolation of enolases A and B by chromatography on triethylaminoethylcellulose. A partially purified mixture of enolases (300 optical density units) was dialyzed against 5×10^{-3} M Tris-acetate, 10^{-4} M EDTA, and 7×10^{-4} M magnesium acetate (pH 8.4) and applied to a 3×10 cm triethylaminoethylcellulose column which was prepared and washed overnight with the same buffer. The enolases were eluted using a three-chambered gradient maker, each chamber containing 400 ml of the dialysis buffer, but made with potassium acetate to 0.025 ionic strength in the middle chamber and 0.5 ionic strength in the chamber farthest from the column. The column was eluted at 0.3 ml/min, and 10-ml fractions were collected. Enzyme assays were at 23°; other conditions were as described in Westhead and McLain (1964). Specific activities are expressed as ΔOD_{230}/min per OD_{280} of enzyme in the assay medium (Westhead and McLain, 1964). The fractions containing enolases A and B (between the arrows) were pooled, dialyzed, and lyophilized before use.

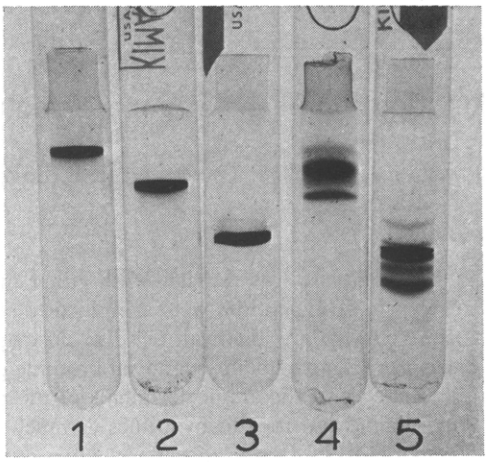

FIGURE 2: Disc electrophoresis of native and cyanogen bromide digested enolase preparations. Tubes 1–3 are from disc electrophoresis of 50 μg of a typical yeast enolase A preparation. The electrophoresis was done at pH 9.5 (tube 1); 8.5 (tube 2) (Hedrick and Smith, 1968); and 2.3 (tube 3). The patterns in tubes 4 and 5 were obtained by disc electrophoresis of 100 μg of cyanogen bromide digest of yeast enolase A at pH 9.5 (tube 4) and 2.3 (tube 5). Tubes 4 and 5 also contained 4 M urea, which was deionized before addition to the other gel reagents. Riboflavin and light were used to polymerize gels containing urea.

added to the enolase solutions to a final weight ratio of 1 to 25–30 parts of enolase in two stages, at zero time and after 3 hr. Digestion was carried out at 37° for a total of 5.5 hr. The digested sample was then acidified with a few drops of glacial acetic acid, and any precipitate was removed by centrifugation at 20,000*g*. Generally, there was little or no precipitate. The centrifuged sample was lyophilized, dissolved in a minimum volume of water, and spotted onto Whatman No. 3MM chromatography paper.

Electrophoresis and chromatography of the tryptic digests were carried out as described by Katz *et al.* (1959) with chromatography in 1-butanol–acetic acid–water (4:1:5) followed by electrophoresis at pH 3.7 in pyridine–acetic acid–water (1:10:289) for 60 or 75 min at 2000 V.

Peptide maps were prepared by dipping the dried sheets in either 0.2% ninhydrin in acetone or in the ninhydrin reagent described by Canfield and Anfinsen (1963). Staining of peptide maps for tryptophan, histidine, and tyrosine was done as described in Bailey (1962). Staining for arginine was done as described by Bennet (1967).

Disc electrophoresis was performed as described in Brewer and Ashworth (1969), based on the work of Ornstein (1964) and Davis (1964). For electrophoresis at pH 8.5, the system of Hedrick and Smith (1968) was used.

Amino acid analyses were performed with a Beckman Model 120C automatic amino acid analyzer on samples of enolase hydrolyzed *in vacuo* for 24, 48, and 72 hr. Tryptophan determinations were performed on the analyzer after hydrolysis in the presence of 4% thioglycolic acid as recommended by Matsubara and Sasaki (1969). Methionine and cysteine were determined as the sulfone and cysteic acid, respectively, after hydrolysis of oxidized enolase prepared by performic acid oxidation by the method of Hirs (1967).

Absorption measurements and enzyme assays were done with a Cary 15 recording spectrophotometer. Determinations of the extinction coefficient of enolase were made using a Beckman Model E analytical ultracentrifuge and interference optics as described by Babul and Stellwagen (1969).

For determination of N-terminal sequences, a quantitative Edman reaction series, and mass spectrometric analysis, a modification of the method of Richards *et al.* (1969) was used (T. Fairwell, R. E. Lovins, F. F. Richards, and W. T. Barnes, manuscript in preparation). A Consolidated Electrodynamics Corp. Model 21-490 single-focussing mass spectrometer equipped with a solid probe inlet was employed for analyses.

Results

To ensure that our measurements of enzyme concentrations were correct, we checked the extinction coefficient of a sample of enolase, comparing its absorption with its refractive increment. The latter was determined in the ultracentrifuge using a synthetic boundary cell and interference optics (Babul and Stellwagen, 1969). The extinction coefficient we obtained was within 1% of the literature value (Warburg and Christian, 1941).

We also obtained fair agreement with the amino acid composition reported by Malmström *et al.* (1959), except for aspartic acid, alanine, half-cystine (see Discussion), and serine (Table I). The half-cystine does not represent a contaminant, since treatment of the enzyme with stoichiometric amounts of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959)

TABLE I: Number of Tryptic Peptides of Enolase Containing Various Amino Acids.

Amino Acid	No. of Residues/Subunit:[a] Malmström *et al.* (1959)	This Work	Peptides Found	Peptides Predicted	
				Identical Subunits	Nonidentical Subunits
Arg	9	10	8–12	9–10	18–20
Lys	27	27	46 ± 2[b]	37–38[b]	74–76[b]
His	7	8	6	7–8	14–16
(Ammonia)	(28)	(24)			
Trp	3	3	3	3	6
Gly	28	26			
Glu	25	23			
Asp	39	33			
Ala	44	36			
Ser	27	20			
Thr	16	13			
Met	4	4			
$^1/_2$-Cys	0	1			
Val	24	25			
Ile	15	15			
Leu	30	27			
Tyr	8	6	7	6–8	12–16
Phe	12	11			
Pro	9	10			

[a] Calculated from the total number of residues per mole of enolase by dividing by two and rounding off to the nearest integer. [b] Total.

in the presence of urea results in inactivation of the enzyme (S. Oh, J. Travis, and J. M. Brewer, unpublished results). We do not know at this time why Malmström *et al.* (1959) or Warburg and Christian (1941) did not observe any half-cystine in yeast enolase. A recovery of 95% of the residue weight was achieved in the amino acid analysis, assuming a subunit molecular weight of 33,600.

There are eight methionines in enolase (Table I), and we would expect five cyanogen bromide peptides from two identical chains and ten peptides from nonidentical ones. Figure 2 shows the patterns obtained on disc electrophoresis in 4 M urea at pH 2.3 and 9.5 of cyanogen bromide digests of yeast enolase. The resolution of the peptides is better at pH 2.3 in urea, and these results will be discussed, although they are consistent with results obtained at the other pH. In general, four major components and a varying number (usually two or three) of minor components are observed. The most slowly migrating of the major peptide components is always the most heavily stained. The two most rapidly migrating major components do not take up much stain, and visibly precipitate in the gel upon removal of the urea in the gels by dialysis. These data suggest that the amino acid compositions of these peptides are very different.

In the figure, the minor components appear to be distinctly thinner than the major ones, and may be aggregates of one or another of the major components. Another possibility is that the major components represent the C-terminal homoserine lactones, and the minor components represent the free acids. The equilibration between the open and closed (lactone) form of homoserine is sufficiently slow to allow separation by electrophoresis (the two forms of the free amino acid can be separated on the ion-exchange resin columns of the equally slow amino acid analyzer). Amino acid analysis of an acid hydrolysate of a similarly treated cyanogen bromide digest showed that little or no methionine remained, so the minor components are not the result of incomplete reaction with cyanogen bromide.

The data in Figure 2 consequently suggest that the subunits are identical, although this is not conclusive. The absence of three to six peptides in the case of nonidentical chains might occur because they are too small to detect by staining, or because they are not resolved. In an attempt to prove or disprove these alternatives, an examination of the amino terminals of the mixture of cyanogen bromide peptides was made. If ten different peptides are formed by reaction of enolase with cyanogen bromide, there is a strong possibility that more than five different amino acids will be obtained as the amino-terminal residues. We treated 0.17 μmole of yeast enolase with cyanogen bromide, then determined the number of moles of N-terminal amino acids in the digest. We obtained only 0.28 μmole of alanine (the amino terminus) and 1.37 μmoles of glycine.

From amino acid analysis of the enzyme (Table I), we found 54 lysines and 20 arginines per mole, which should give 76 tryptic peptides if the two subunits are entirely different, or 38 peptides if they are identical. The results of a typical analysis of tryptic peptides of yeast enolase by peptide mapping are shown in Figure 3a. The peptides were stained with

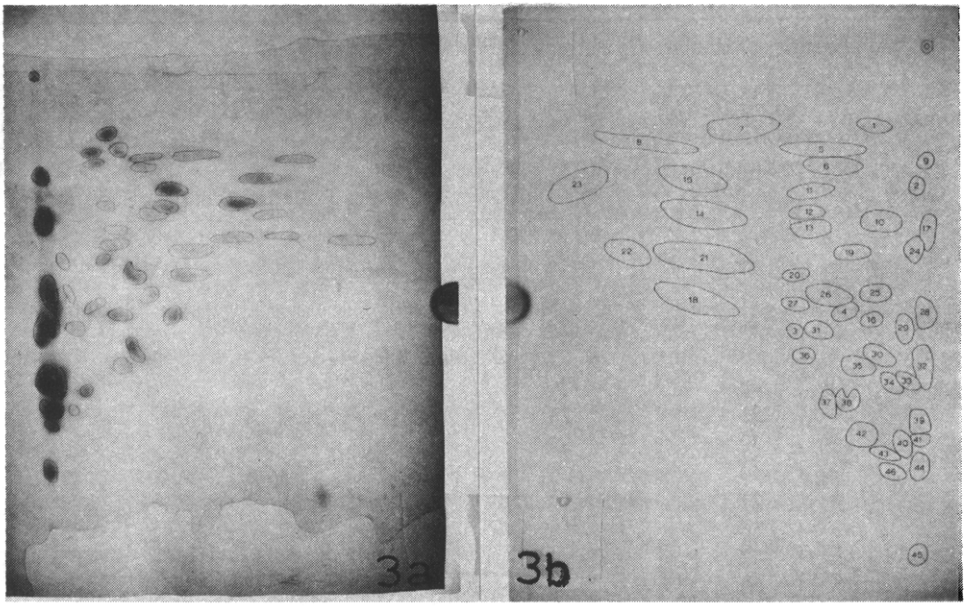

FIGURE 3: Peptide maps of tryptic digests of yeast enolase. (a) Typical peptide map: 2.5 mg of enolase digest was applied to the origin, then chromatographed and electrophoresed as described in Materials and Methods. The paper was stained with the ninhydrin–acetone reagent (*cf.* Materials and Methods), dried in a hood, and placed in a 37° room for 10 min to accelerate color development. (b) Composite peptide map: the data are taken from five maps stained with ninhydrin, three stained or overstained for tyrosine and histidine, three overstained for tryptophan, and two stained for arginine. The location of peptides containing the indicated amino acid is given by their numbers: tryptophan: 5, 31, 44; arginine: (definite stains) 17, 20, 27, 28, 31, 32, 39, 40, (possible stains) 19, 24, 44, 45; histidine: 19, 30, 32 (intense color), 38,39, 40; tyrosine: 8, 11, 12, 14, 15, 26, 31; free lysine or arginine: 45; and N-terminal glycine: 37. The direction of electrophoresis is down (toward the cathode) in both parts a and b; the direction of chromatography is to the right in the case of part a and to the left in the case of part b.

ninhydrin. We made five such maps, which were spotted with 1–4 mg (0.013–0.052 μmole) of enolase digest. The number of peptides found were 44, 46, 45, 45, and 48, for an average of 46 ± 2 peptides.

Incomplete digestion by trypsin would produce a larger number of peptides than the lysine and arginine content would predict. To determine whether the trypsin digestion was complete, a sample of the tryptic peptides of enolase was redigested with carboxypeptidase B essentially as described by Cardenas and Wold (1968), except that digestion was done for as long as 5 hr. During the carboxypeptidase digestion, the equivalent of about 18[1] of the 27[1] lysines and all 10[1] of the arginines were released within 30 min. Another 6[1] lysines were released more slowly over the next 4.5 hr, leaving 3[1] bonds which are either more resistant to carboxypeptidase B or which were not broken by the initial trypsin digestion (see Discussion).

Identical maps of tryptic peptides were stained for the presence of tryptophan, tyrosine, histidine, and arginine. Table I gives the amounts of these amino acids in yeast enolase, the number of peptides found with those amino acids, and the number expected for identical and nonidentical chains. It is clear that the results are more consistent with two identical subunits than two different ones. Only the number of arginine peptides is possibly greater than the number predicted for identical chains, and the existence of lysylarginine, arginyllysine, or arginylarginine sequences would account for this (see Discussion). A lower number of histidine-containing peptides than predicted in either case is possibly the result of two or three of the residues being located in a single peptide. One histidine-containing peptide stains considerably more intensely than the others. The composite peptide map (Figure 3b) shows the locations of all the residues stained for.

We have reexamined the question of how many N-terminal residues there are in the enzyme, using a modification of the quantitative N-terminal Edman reaction described by Richards *et al.* (1969). We treated 0.64 μmole of yeast enolase, as determined from the absorption of the enzyme solution and the extinction coefficient of Warburg and Christian (1941), with methyl isothiocyanate. We obtained 1.13 ● 0.11 μmoles of N-terminal alanine methylthiohydantoin, which is 1.76 ± 0.19 moles of N-terminal amino acid per mole of protein (Figure 4). On removing the alanine methylthiohydantoin and again treating the protein with the Edman reagent, 0.96 μmole of glycine methylthiohydantoin was obtained as the second residue, which is 1.44 moles of amino acid/mole of enolase. Since some loss of protein occurs during the extractions of the derivatives, a relatively smaller recovery of the second amino acid is expected. No other amino acid was detected in either determination. A further analysis of the N-terminal sequence of the protein showed that the sequence for both chains of enolases A and B is: Ala-Gly-Lys-Val-Gly-Asp-Thr-Glu(NH_2).

Preliminary data suggest that the next four residues are: Lys-Pro(Ala?)-Ile(Val?)-Gly. This sequence work confirms and extends the results of Malmström *et al.* (1959) who found the N-terminal amino acid sequence of a mixture of enolases A and B to be Ala-Gly-(Val,Lys). We are now in the process of obtaining the complete amino acid sequence of

[1] Per subunit.

yeast enolase. However, the results cited show that the two chains are chemically identical for at least the first eight residues. In addition, the last (C terminal) residue was shown to be leucine by Nylander and Malmström (1959), using carboxypeptidase A digestion. This finding has been confirmed in this laboratory (J. Hancock and J. M. Brewer, unpublished results).

Discussion

The fact that only alanine and glycine are obtained as the amino-terminal residues of the cyanogen bromide fragments and the stoichiometry observed indicate that no cleavage of the enzyme after aspartyl residues had occurred during digestion. Such cleavage has been observed in acid solutions (Schultz, 1967). The stoichiometry also suggests that we had obtained complete cleavage at the methionine residues.

The observation that we obtain an intermediate number of tryptic peptides on mapping might be interpreted as indicating that some regions of the subunits are different. Incomplete digestion by trypsin would also produce such a result. The carboxypeptidase data suggest that the latter may be the case. However, it is known (Van Orden and Smith, 1954) that some peptide bonds such as prolyllysine, glutamyllysine, or aspartyllysine are more resistant to digestion with carboxypeptidase B, so the failure of carboxypeptidase B to release all the lysines and arginines within 30 min is easily explained. The lack of precipitate occurring upon acidification of the tryptic digest also suggests that digestion is completed under our conditions.

It is also known (Neurath, 1960) that lysyl–proline bonds are not broken by trypsin, and we have preliminary evidence that the ninth and tenth residues of enolase may be lysine and proline. So at least one such trypsin-resistant bond probably exists in the enzyme. The existence of three such bonds would decrease the number of tryptic peptides expected on peptide mapping by 3.

On the other hand, about 1.5 residues of lysine and 0.5–1.5 residues of arginine were found in the tryptic digests before carboxypeptidase treatment, which demonstrates the presence of an unknown number of arginyl–lysine, arginyl–arginine, lysyl–lysine, or lysyl–arginine bonds in enolase. Since tryptic digestion of these bonds would be expected to result in a heterolytic cleavage (Neurath, 1960), some additional peptides would be produced.

An excess of peptides might also result from traces of chymotryptic activity in the trypsin. However, the absence of such activity upon direct assay (see Materials and Methods) and the fact that in addition the trypsin was treated to remove any such activity make this possibility unlikely. Consequently, we feel that the subunits are probably identical.

The failure of Malmström *et al.* (1959) to detect two amino terminals is not difficult to understand. Those authors used 1,2,4-fluorodinitrobenzene to measure the number of amino terminals in enolase. This method is liable to errors from several sources, and most of these would tend to produce an underestimate of the number of residues. The sensitivity of the mass spectrometric analysis used in this paper is such (0.01 μmole of any amino acid can be identified) that the failure to detect any other amino acids in the sequence analysis shows that the protein preparation used must be at least 95% pure. Since the Edman reaction used in this paper is known to go to at least 98% of completion (Richards *et al.*, 1969), the superiority of this method is manifest. In general, any errors in quantitation beyond ±10% would likely arise from the presence of some ultraviolet-absorbing impurity in the protein preparation, or to an erroneous extinction coefficient.

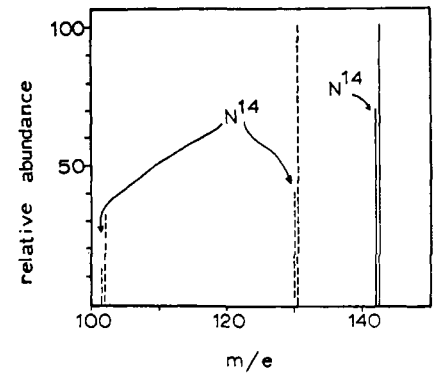

FIGURE 4: Mass spectra of N-terminal and penultimate amino acid methylthiohydantoins. The N-terminal amino acid is alanine (solid lines); no other amino acid derivatives were detected. The second amino acid is glycine (dashed lines); again, no other amino acid derivatives were detected. The *m/e* = 130/131 ions are the molecular ions of the ^{14}N- and ^{15}N-labeled glycine methylthiohydantoins. The ions at *m/e* = 102/103 represent ions which have lost C=O (M = 28) from the molecular ions in each case. The amounts of the amino acids were determined by isotopic dilution, using ^{15}N-labeled alanine and glycine methylthiohydantoins as described by Richards *et al.* (1969). "^{14}N" refers to the relative amounts of the natural amino acids.

Cardenas and Wold (1968) showed that the two chains of rabbit muscle enolase were also identical or nearly so, and that there were two binding sites for a competitive inhibitor. Since magnesium has a structural function in the rabbit muscle enzyme also (Winstead and Wold, 1965), it is tempting to suggest that there may be two substrate binding sites in the yeast enzyme as well. Indeed, there is some preliminary evidence that this is the case (S. Oh and J. M. Brewer, unpublished data). If this is so, one wonders why the two most tightly bound magnesium ions tend to have separate functions (Brewer and Weber, 1965, 1966; Hanlon and Westhead, 1969a,b).

The unusually high specific activities obtained by us are puzzling, but reproducible. We believe the enzyme of lower specific activity prepared by Westhead and McLain (1964) is essentially chemically pure; the only apparent difference between their preparations and ours is the turnover number. Recently, Hanlon and Feins (1969) reported kinetic evidence which can be interpreted as indicating that enolase as prepared by the method of Westhead and McLain (1964) has two active sites which have different Michaelis constants for magnesium and turnover numbers which differ by a factor of 4. An alternative hypothesis is that their enzyme consists of a population of two kinetically distinguishable types of enolase (perhaps also distinguishable by the presence of cysteine or cystine?). The magnesium Michaelis constants correspond to the two lowest magnesium–enolase dissociation constants, measured by equilibrium dialysis (Hanlon and Westhead, 1969a,b). The observations of Brewer and Weber (1966), Hanlon and Westhead (1969a,b), and Hanlon and

Feins (1969) are clearly more consistent with different subunits than identical ones. However, our data suggest the possibility that some aspect of our treatment of the enzyme, possibly involving the chromatography on triethylaminoethylcellulose, "activates" the sites with the lower turnover number mentioned above. We are investigating these hypotheses.

Acknowledgments

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