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Studies on Rabbit Muscle Enolase. Evidence for Two Identical Polypeptide Chains and Two Substrate Binding Sites in the Active Enzyme*

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ABSTRACT: Rabbit muscle enolase, an enzyme containing two polypeptide chains of 41,000 molecular weight/active unit of 82,000 molecular weight, was treated with trypsin and with cyanogen bromide, and the number of peptides produced in each treatment was estimated from the number of spots of peptide maps and one-dimensional electrophoretograms, respectively. The extent of the trypsin digestion was assessed by the liberation of carboxyl-terminal lysine and arginine residues susceptible to attack by carboxypeptidase B, and the extent of the cyanogen bromide reaction was determined by methionine analyses. Both the total ninhydrin-positive peptides and the arginine-containing peptides (visualized with Sakaguchi reagent) were counted. All the results are consistent with a unique polypeptide sequence of one-half the molecular weight of the active enzyme. It is thus concluded that the two polypeptide chains in rabbit muscle enolase are identical. The number of substrate binding sites in rabbit muscle enolase was determined by gel filtration techniques, using the substrate analog [^{14}C]glycolic acid phosphate as the ligand. Analog (2 moles) was bound per 82,000 g of enzyme, with an average dissociation constant of 2×10^{-4} M. This agrees reasonably well with the K_I (competitive) value of 7×10^{-4} M for glycolic acid phosphate.

Earlier studies on rabbit muscle enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) have established that the active enzyme (mol wt 82,000) is made up of two polypeptide chains of 41,000 molecular weight (Winstead and Wold, 1965). Both polypeptide chains have an amino-terminal *N*-acetylalanine and the carboxyl-terminal tripeptide sequence lysyl-alanyl-lysine (Winstead and Wold, 1964). In view of these similarities, the studies reported in this paper were undertaken in an attempt to establish whether or not the entire amino acid sequence is identical in the two chains.

To this end, the enzyme was degraded with cyanogen bromide and trypsin and the number of resulting peptides was determined by peptide mapping. In order for this approach to have any validity, it is essential that both the extent and the specificity of the peptide cleavage be evaluated. This is readily accomplished for the cyanogen bromide reaction, since an amino acid analysis of the acid-hydrolyzed reaction product will give the extent of the reaction in terms of conversion of methionine into homoserine (and homoserine lactone) (Gross and Witkop, 1962) and at the same time, indicate the specificity, in terms of the recovery of other potentially reactive amino acids. For the tryptic digestion, no such direct assay is available, however. In this work we took advantage of the specificity of carboxypeptidase B for basic amino acid residues, and monitored the trypsin digestion both for liberation of carboxyl-terminal lysine and arginine susceptible to attack by carboxypeptidase B, and for free lysine and arginine liberated directly by trypsin. With this type of information at hand, the total number of peptide bonds broken, and thus also the total number of peptides expected if the chains were either identical or different, can readily be estimated quite accurately.

When the results from the peptide counts showed that rabbit muscle enolase consists of two identical polypep-

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tide chains, it was felt of interest to establish the number of substrate binding sites associated with this active two-chain structure. The binding of a substrate analog, glycolic acid phosphate, to enolase was determined by gel filtration and the results demonstrate that the native enzyme (mol wt 82,000) has two substrate binding sites.

Experimental Section

Materials and Assays. Enolase was isolated from frozen mature rabbit muscle (Pel-Freez Biologicals, Inc., Rogers, Ark.) according to the method of Winstead and Wold (1966). The enzyme was recrystallized at least three times and its purity was established by the criteria of specific activity and starch gel electrophoresis.

Enolase activity assays were performed using the spectrophotometric method of Warburg and Christian (1942) with the water-soluble cyclohexylammonium salt of the substrate (D-glyceric acid 2-phosphate) and a Zeiss PMQ II spectrophotometer with the temperature controlled at 25°, according to previously published procedures (Winstead and Wold, 1966).

Comparison of Peptide Chains. Enzymatic Digestion. Solutions of enolase were alternately acidified and made alkaline by dropwise addition of concentrated HCl and NH_4OH until the protein was denatured and had precipitated. The suspension was neutralized and the denatured protein was collected by centrifugation, washed twice with glass-distilled water, and then suspended in a sufficient volume of 0.1 M ammonium bicarbonate (pH 8.0) to give a 10-mg/ml protein concentration. A drop of phenol red was added as indicator. An amount of trypsin (Pentex, Inc.) equal to 1% the weight of the enolase was added and the incubation mixture was stirred at 37°. The solutions were generally clear after 30-min incubation, but the digestions were continued for at least 2 hr. At the end of the digestion period, 2- and 4-mg samples were removed and lyophilized for peptide mapping. At the same time, 3–5-ml samples (30–50 mg of peptides) were boiled for 15 min to inactivate the trypsin and were then lyophilized. This material was subsequently used for the carboxyl-terminal analyses with carboxypeptidase.

The lyophilized tryptic peptides (30–50 mg) were dissolved in sufficient 0.05 M potassium bicarbonate (pH 7.6) to make a 5-mg/ml solution. A zero-time sample (0.30 ml) was removed before carboxypeptidase was added. Sufficient carboxypeptidase B (DFP[1]-treated, frozen solution, Worthington Biochemical Corp.) was added to the tryptic peptides to give a weight ratio of 1:100 and the digestion was carried out at 37°. At various times during the digestion, 0.3-ml samples (1.5 mg of peptides) were removed and the digestion was stopped by adding an equal volume of 10% trichloroacetic acid at 0°. Insoluble material was removed by centrifugation (10,000*g* for 15 min at 0°). The precipitate was washed with 0.6 ml of 5% trichloroacetic acid, centrifuged as before, and the supernatant was added to that from the first centrifugation. The supernatants were lyophilized and stored at −20° for amino acid analysis (on the Spinco Model 120C amino acid analyzer). When a solution of carboxypeptidase B alone was precipitated with trichloroacetic acid and treated in the same manner as the samples from the digestion mixture, only negligible amounts of free amino acids were found. A 0.3-ml sample of each digestion mixture was also subjected to acid hydrolysis (6 N HCl, 21 hr at 110°) in sealed, evacuated vials to allow determination of the total amount of amino acids (primarily lysine and arginine) in the incubation mixture, and the release of amino acids from the carboxypeptidase digestion could then be accurately calculated as per cent of this total.

Cyanogen Bromide Degradation (Gross and Witkop, 1962). Rabbit muscle enolase was dialyzed overnight against 0.01 M ammonium bicarbonate and then for 2 hr against glass-distilled water. A 30-fold molar excess (based on methionine content of the enzyme) of CNBr was dissolved in a volume of 0.3 N HCl equal to the volume of the protein solution. The protein solution was added rapidly with stirring to the CNBr solution and the reaction mixture was left with stirring at room temperature for 24 hr. The reaction was stopped and volatile components were removed by lyophilization (three times). The extent of the cleavage of methionyl peptides was determined by amino acid analysis on the Spinco Model 120C amino acid analyzer after acid hydrolysis. In order to eliminate possible ambiguities due to sulfhydryl oxidation and disulfide interchange (enolase contains 12 moles of SH groups/mole (Malmström, 1962)), a fraction of the cyanogen bromide peptides was always oxidized with performic acid at −7° (Hirs, 1956) and compared with the unoxidized peptides in the subsequent electrophoresis runs.

Peptide Mapping. Peptide maps of the tryptic peptides were made on Whatman No. 3MM filter paper, 35 × 40 cm. Samples (2–4 mg) of lyophilized tryptic peptides were dissolved in 0.10 ml of electrophoresis buffer, and the paper was spotted with as much of the peptide solution as possible. Electrophoresis was carried out in pyridine acetate buffer (pH 6.4) (pyridine–glacial acetic acid–water, 100:4:900, v/v) at 1800 V for 90 min, using a Brinkmann Pherograph electrophoresis apparatus. After electrophoresis, descending chromatography was carried out in the second dimension, using the solvent system 1-butanol–glacial acetic acid–water (4:1:5, v/v). Peptides were detected either by dipping in ninhydrin–collidine reagent (Canfield and Anfinsen, 1963) or by spraying with Sakaguchi reagent (Elliott, 1962).

The cyanogen bromide peptides were investigated by one-dimensional electrophoresis only. Because of solubility problems, the number of practical electrophoresis systems was limited. Using the criterion of complete removal of peptides from the point of origin after electrophoresis as an evaluation of different media and buffers, starch gel electrophoresis (Tsuyuki, 1963) with pyridine acetate buffer (pH 6.5) (pyridine–acetic acid–water, 10:1:109) and cellulose electrophoresis on acetate strips with Tris-citrate–urea buffer (pH 5) (0.05 M Tris and 3 M urea, titrated to pH 5 with 1 M citric acid) and with sodium citrate–urea buffer (pH 5) (0.01 M sodium citrate and 0.5 M urea) were selected. All the elec-

[1] Abbreviations used are listed in *Biochemistry 5*, 1445 (1966).

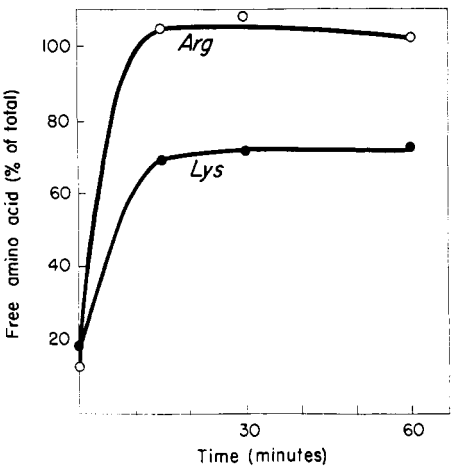

FIGURE 1: The rate of carboxypeptidase B catalyzed release of arginine and lysine from the tryptic peptides of rabbit muscle enolase. The digestion was carried out at 37° in 0.05 M $KHCO_3$ buffer (pH 7.6) with a weight ratio of carboxypeptidase to peptides of 1:100. The results are given as per cent of the total, the total (100%) being the value obtained by analyses of acid-hydrolyzed samples of the peptide mixture. The zero-time sample was removed prior to the addition of carboxypeptidase and represents arginine and lysine released by trypsin alone.

trophoretograms were stained with dye, and in the case of the cellulose acetate runs, parallel strips were also developed with ninhydrin spray to ensure detection of any component too small to stain with the dye.

Binding Studies with Glycolic Acid Phosphate. [1-^{14}C]-Glycolic acid phosphate (8.6×10^4 dpm/μmole) was prepared and generously provided by Dr. F. C. Hartman. Glycolic acid phosphate is a competitive inhibitor for rabbit muscle enolase, having a K_I of 7.1×10^{-4} M (F. C. Hartman, personal communication).

Binding studies with the substrate analog were performed by the method of Castellino and Barker (1966). A 0.8×100 cm column was packed with Bio-Gel P-6 (Bio-Rad Laboratories) which had been previously washed with 25% methanol, deionized water, glass-distilled water, 0.5 M KCl, and several hold-up volumes of imidazole–KCl buffer (0.05 M imidazole, 0.2 M KCl, and 10^{-3} M $MgSO_4$, pH 6.95). The column was finally equilibrated at 25° with the desired concentration of [^{14}C]glycolic acid phosphate in the same imidazole–Mg–KCl buffer (equilibration solution).

A total quantity of 33–41 mg (0.4–0.5 μmole) of rabbit muscle enolase was dialyzed against the imidazole–Mg–KCl buffer. Immediately before applying the enzyme solution to the column, sufficient concentrated [^{14}C]glycolic acid phosphate solution was added to the enzyme solution so that the concentration of [^{14}C]glycolic acid phosphate in the enzyme solution would equal that in the equilibration solution. The final protein concentration applied to the column was $1–2 \times 10^{-4}$ M (8–16 mg/ml). The enzyme was eluted with the appropriate equilibration solution at a flow rate of 0.4 ml/min, and 1.3-ml fractions were collected. An LKB Uvicord was useful in determining the location and shape of the protein peak.

The protein concentration in the collected fractions was determined from the absorbance at 280 mμ in comparison with weighed samples of pure enolase. Enolase activity assays of the protein fractions were also performed to determine whether loss of activity had occurred during the gel filtration experiment.

The amount of free and bound glycolic acid phosphate was determined from the distribution of radioactivity in the elution pattern. The radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer. Samples (20 μl) were measured into glass scintillation vials containing 1 ml of Hyamine hydroxide 10X (Packard Corp.) and 15 ml of scintillation fluid[2] was then added.

Results

Comparison of the Peptide Chains of Rabbit Muscle Enolase. Rabbit muscle enolase contains 68 moles of lysine and 30 moles of arginine per 82,000 g of enzyme (Holt and Wold, 1961). Since the two carboxyl-terminal residues are lysine (Winstead and Wold, 1964), the total number of tryptic cleavages should be two less than the total number of arginine and lysine residues.

Typical amino acid release curves for arginine and lysine during carboxypeptidase B attack on the mixture of tryptic peptides from enolase are given in Figure 1. The analyses of the zero-time samples (sample of tryptic peptides removed before carboxypeptidase was added) show that 18% of the total lysine (12 moles/mole) and 10% of the arginine (3 moles/mole) were liberated by trypsin treatment alone. The presence of these quantities of free lysine and arginine must be accounted for in estimating the theoretical number of peptides.

Figure 1 also shows that by the criterion of rapid carboxypeptidase B release, all the arginine bonds have been cleaved by trypsin. Only 73% of the lysine residues was released by carboxypeptidase B, however, and this introduced another ambiguity in the calculation of the lysine peptides. The missing 27% of the lysine residues may have been resistant to the initial trypsin attack or they may have been released completely by trypsin and for some reason been resistant to carboxypeptidase attack. Any combination of incomplete attack by both trypsin and carboxypeptidase between these two limits must obviously also be considered.

Based on these considerations the following predictions can be formulated for the number of tryptic enolase peptides (30 moles of arginine and 68 moles of lysine per mole). Cleavage (100%) at 30 arginine residues, 3[3] liberated as free arginine by trypsin, should give 28 arginine spots (27 arginine peptides plus free arginine) for nonidentical chains and 14–15 spots (13–14 arginine

[2] 2,5-Diphenyloxazole (8 g) and 2-*p*-phenylenebis(5-phenyloxazole) (200 mg) were dissolved in 1 l. of toluene. Four parts of this toluene solution were mixed with three parts of absolute ethanol for use as scintillation fluid.

[3] Although two identical chains should give only even numbers in these analyses, the odd numbers are used whenever they occur without any attempt at corrections. In view of the total errors involved in this type of "numerology" such corrections would be as ambiguous as the odd numbers.

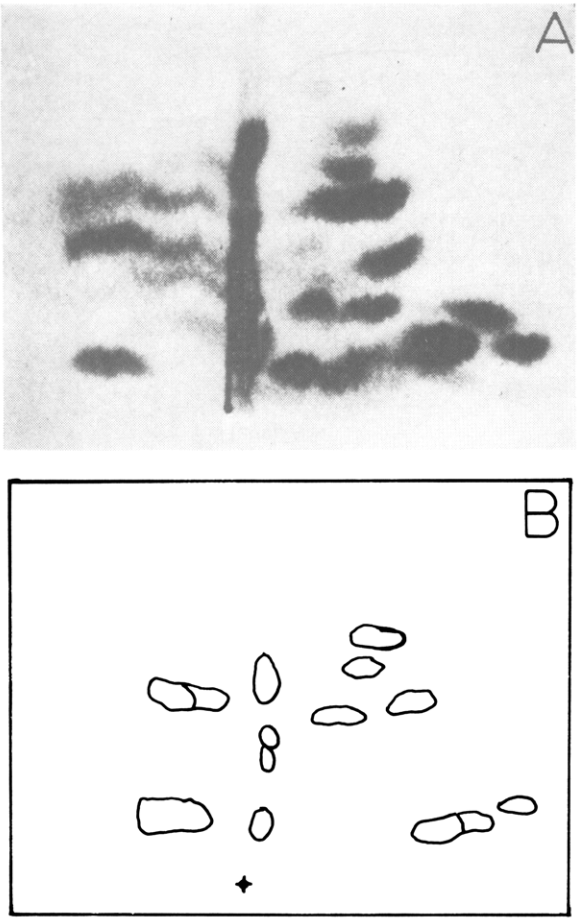

FIGURE 2. Peptide maps of tryptic peptides from enolase. A was developed with ninhydrin and B with Sakaguchi reagent. Because of the instability of the color in the latter map, the spots were outlined with pencil immediately after color development.

peptides plus free arginine) for identical chains. Assuming 73% tryptic cleavage at (68–2) lysine residues gives 48 lysine cleavages, 12 of which resulted in free lysine, and this should give 38 spots (37 peptides plus free lysine) for nonidentical chains and 20 spots (19 peptides plus free lysine for identical chains. This gives a total of 66 ninhydrin spots (28 + 38) for nonidentical and 34–35 ninhydrin spots (14–15 + 20) for identical chains. These are the minimal values assuming 100% carboxypeptidase release of all carboxyl-terminal lysines produced by 73% lysine cleavage by trypsin. (This is probably not a good assumption, as it is already known that the third residue residue from the carboxyl-terminal end of rabbit muscle enolase is a lysine residue which is resistant to carboxypeptidase B action; Winstead and Wold, 1964.) If, on the other extreme, one assumes that the trypsin digestion of lysine bonds was complete, maximal values are obtained. This will give 58 lysine spots (57 peptides plus free lysine) for nonidentical chains and 30 spots (29 peptides plus free lysine) for identical chains. The maximum number of total ninhydrin spots, assuming complete tryptic cleavage, would thus be 82 and 44–45 for nonidentical and identical chains, respectively.

Since the carboxypeptidase experiments unequivocally showed complete arginine release, one would therefore in summary predict 28 or 14–15 arginine peptides for nonidentical and identical chains and a total number of ninhydrin spots between the limits of 67 and 82 for nonidentical chains and between 35–36 and 44–45 for identical chains.

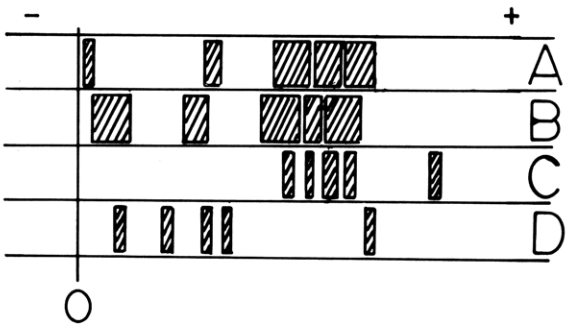

FIGURE 3: Tracings of typical electrophoretic peptide patterns of cyanogen bromide treated enolase. (A and B) Starch gel electrophoresis of peptides in pH 6.5 pyridine acetate buffer at 200 V for 8 hr. (A) Before oxidation. (B) After performic acid oxidation. The gel was developed with Amido Black. (C and D) Cellulose acetate strip electrophoresis at pH 5. (C) In Tris–citrate–urea buffer, 400 V for 2.5 hr. (D) Sodium citrate–urea buffer, 200 V for 1.4 hr. The strips were developed with ninhydrin. Experimental details are given in the text.

Nonspecific or incomplete cleavages would give higher values for both arginine and total ninhydrin peptides. Attempts to assess the extent of nonspecific cleavages by carboxypeptidase treatments were complicated by the lack of absolute specificity in both carboxypeptidases A and B. In addition to lysine and arginine only tyrosine and phenylalanine were released in significant amounts, indicating that some chymotryptic cleavages may have occurred. If this is so, it would not affect the number of arginine peptides, but would make the number of ninhydrin peptides too high. Figure 2 shows typical tryptic peptide maps of enolase, and the 40–45 ninhydrin peptides and 13–15 arginine peptides which were consistently found in these maps are in excellent agreement with the numbers predicted for identical polypeptide chains.

Cyanogen Bromide Peptides. In spite of some ambiguities in the analyses of the cyanogen bromide peptides, the results from these studies support the conclusion that the chains are identical. Enolase contains 12 moles of methionine/82,000 g of protein (Holt and Wold, 1961) and a maximum of 7 cyanogen bromide peptides should thus be produced from two identical chains and 14 from two nonidentical chains. The recovery of methionine after the cyanogen bromide reaction varied from 10 to 20% in three experiments, and the reaction was thus only 80–90% complete. If this is due to the presence of one or two specific methionyl peptides which are relatively resistant to cyanogen bromide, a lower number of peptides would result. If, on the other hand, the intact methionyl peptide bonds are randomly distributed, the number of peptides should be high. The electrophoretic patterns in Figure 3 are typical of the results obtained. In all the systems used, the highest number of

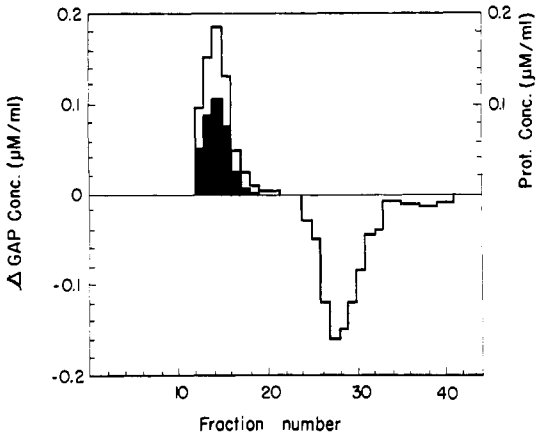

FIGURE 4: Typical elution pattern from a gel filtration column of Bio-Gel P-6 equilibrated with a solution of [1-^{14}C]-glycolic acid phosphate (5.8×10^{-4} M), imidazole (0.05 M), $MgSO_4$ (10^{-3} M), and KCl (0.2 M) at pH 6.95. Rabbit muscle enolase (40.1 mg) in approximately 3 ml of the equilibration solution was put on the column and eluted with equilibration solution at a flow rate of 0.4 ml/min. Fractions of 1.3 ml were collected. Open bars: ΔGAP concentration in micromoles per milliliter (concentration of total glycolic acid phosphate (GAP) concentration minus free glycolic acid concentration (base-line value)); darkened bars, protein concentration.

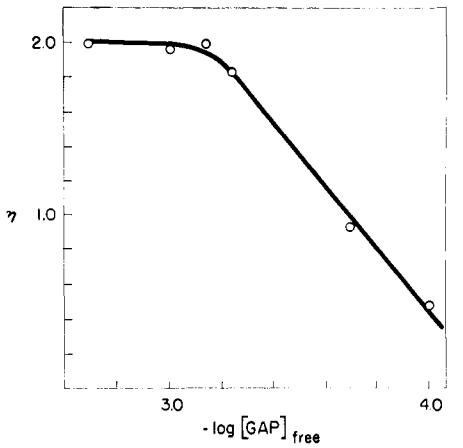

FIGURE 5: Plot of glycolic acid phosphate binding to rabbit muscle enolase. n is the number of moles of glycolic acid phosphate bound per mole of enzyme. $[GAP]_{free}$ is the concentration of glycolic acid phosphate in the equilibration solution used in each experiment. Experimental details are given in the text.

components observed was 5, and the observed number was the same before and after performic acid oxidation and with both dye and ninhydrin development.

The Number of Substrate Sites in Rabbit Muscle Enolase. A typical elution diagram for the gel filtration binding studies with [1-^{14}C]glycolic acid phosphate is given in Figure 4, and the results from six such runs plotted in terms of n, the number of moles of glycolic acid phosphate bound per mole of protein, *vs.* the negative log of the free glycolic acid phosphate concentration, are given in Figure 5. The data show that under the conditions of the experiment, at least 2 moles of glycolic acid phosphate can be bound/mole of rabbit muscle enolase, calculated from a molecular weight of 82,000 for rabbit muscle enolase and a molecular weight of 453 for glycolic acid phosphate (tricyclohexylammonium salt). No loss of enolase activity was encountered in the course of the gel filtration runs, so the possibility of nonspecific binding to an inactive form of the enzyme can be disregarded. The average dissociation constant, 2×10^{-4} M, obtained from the data in Figure 4 is similar enough to the kinetically determined dissociation constant, $K_I = 7 \times 10^{-4}$ M (F. C. Hartman, personal communication) to conclude at least tentatively that the enzyme has two sites at which the analog competes with the substrate for binding.

Discussion

The results from previous comparative studies of enolases from different biological sources have demonstrated significant variations in the structural properties of the different enzymes, and yet have not revealed any major differences in catalytic parameters (Cardenas *et al.*, 1967; Cory and Wold, 1966). The tentative conclusion of these results has led to the working hypothesis that the active enolase site has remained remarkably constant through evolution and that a detailed comparative study of the different proteins carrying this active site should uncover a set of chemical properties common to all enolases and representing the relevant active site components. Further studies are required to test this hypothesis and the results reported in this paper represent the first step toward better defined molecular models of enolases.

All the results from the peptide mapping and counting are consistent with an enzyme of 82,000 molecular weight made up of two identical polypeptide chains. The number of arginine peptides produced by tryptic digestion is exactly that predicted for identical chains, and in spite of some ambiguities in the total number of tryptic peptides and in the number of cyanogen bromide peptides this data can also best be interpreted in terms of identical chains. The use of carboxypeptidase digestion as a method for monitoring the extent of the trypsin digestion appears practical and in this work supplied information essential for the interpretation of the results.

In the determination of the number of substrate binding sites, it was felt important to avoid the hazards of working with a dynamic equilibrium of substrate and product in the gel filtration experiments. For this reason, the catalytically inert substrate analog, glycolic acid phosphate, was used in the binding studies. The validity of the conclusion from these experiments, that rabbit muscle enolase contains two substrate sites, therefore clearly depends upon the validity of the assumption that the experimentally established two analog binding sites correspond to two substrate binding sites. Based on the structural similarity between substrate (glyceric acid 2-phosphate) and analog (glycolic acid phosphate) and on the fact that the analog is a simple competitive inhibitor of rabbit muscle enolase, this assumption seems quite reasonable. However, having established the existence

of two substrate binding sites does not necessarily mean that the two sites are catalytically active. Especially in view of the proposed dual role of magnesium ion as a structural component as well as a catalytic participant in both yeast enolase (Brewer and Weber, 1966) and in rabbit muscle enolase (Winstead and Wold, 1965), it is quite possible that an activating, but noncatalytic substrate binding site could be present in the enzyme. The available data cannot establish to what extent the two sites interact, or whether they are catalytically active sites.

The results reported here together with those reported previously (Winstead and Wold, 1964, 1965) in conclusion lead to the following picture of rabbit muscle enolase. The enzyme (82,000 molecular weight) consists of two identical polypeptide chains stabilized in the dimer form by magnesium ion. The dimer has two substrate binding sites, but no catalytic activity has ever been found associated with the single individual chains (Winstead and Wold, 1965).

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