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ORIGIN OF MULTIPLE SPECIES OF YEAST ENOLASE A ON ISOELECTRIC FOCUSING

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SUMMARY: On isoelectric focusing, bakers yeast enolase A has been shown to resolve into one major and at least two minor species. Refocusing of individual species isolated by electrofocusing shows the minor species to be formed from the major one. The extent of formation of the minor species increases with electrofocusing time and decreasing quantity of protein electrofocused, suggesting that the protein, on dissociating, tends to change into forms(s) with more alkaline isoelectric points.

INTRODUCTION

Westhead and McLain (1) showed that allowing yeast to age at 4° or prolonged autolysis produced additional species of enolase (called B, C, etc.) which could be separated from the presumably native "A" form by chromatography on TEAE cellulose. This heterogeneity was ascribed to deamidation of the A enzyme.

Isoelectric focusing of unchromatographed yeast enolase by Susor et al. (2) produced at least seven components including two major fractions. Even after chromatography on TEAE cellulose, the "A" fraction could be resolved into a main peak and at least two other minor species. Brewer et al. (3) showed the A enzyme to consist of two identical subunits, but Oh et al. (4) obtained evidence for possible sequence heterogeneity about the single cysteine per subunit. Blomquist (5) has also found three enolase "isozymes" with different isoelectric points.

Because of its importance to the sequence work on the enzyme which is now under way (6,7), we reexamined this question and have obtained evidence that the species of enolase A aside from the major peak are artifacts, resulting from some process apparently connected with dissociation of the enzyme.

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MATERIALS AND METHODS

The enolase used in these studies was the A form of the enzyme, isolated from bakers yeast using a modification of the method of Westhead and McLain (1). Both "recycled" and "fresh" enzyme preparations were examined. The "fresh" preparation was deionized before use as described by Faller and Johnson (8), then concentrated to 120 mg/ml by pervaporation. The enzyme was assayed as described by Westhead and McLain (1), using a Spectronic 200 spectrophotometer (Bausch and Lomb) at 230 nm. All assays were done at room temperature (22-25°). All isoelectric focusings were done at 4°. Collected fractions were stored at 4°.

The electrofocusing apparatus and ampholyte (pH 5-7 range) were from LKB Instruments. Measurements of pH were made at room temperature using a Corning Model 10 pH meter.

Sucrose was "density gradient grade" from Schwarz/Mann Inc. 2-D-phosphoglycerate was purchased as the barium salt from Calbiochem and was crystallized as the tricyclohexylammonium salt.

RESULTS

28 mg of enzyme was focused 45 hours. On elution, we obtained one major peak with pI = 6.1, a secondary peak at pH 6.3 and some additional active material with an isoelectric point in the pH 7 range (Figure 1). The major and secondary species had nearly the same specific activities. It was not possible to determine the true specific activity of the more alkaline material, as the absorbance of the ampholyte increases rapidly in that region. The focusing was repeated twice, using 44 mg of enzyme each time, focused for 45 and for 67 hours. The same pattern was obtained. The peak fraction from the major and secondary species was set aside after each focusing. Three peak fractions of each species were then pooled, dialyzed against 0.01 ionic strength tris-HCl, pH 7.8, with 0.1 mM magnesium chloride for 2 days, then against several changes of water for 2 days. Then the major peak material and secondary peak material were separately refocused, each for 45 hours.

The refocused primary peak fractions again gave a pattern exactly like the original material. The refocused secondary peak fractions gave only a small peak at pI = 6.3, together with some pH 7 material.

Two focusings of 44 mg each of a "fresh" preparation of enzyme (deionized beforehand), both for 45 hours, each again gave the same pattern (not shown). In one case, excess magnesium was added to the enzyme before focusing; this

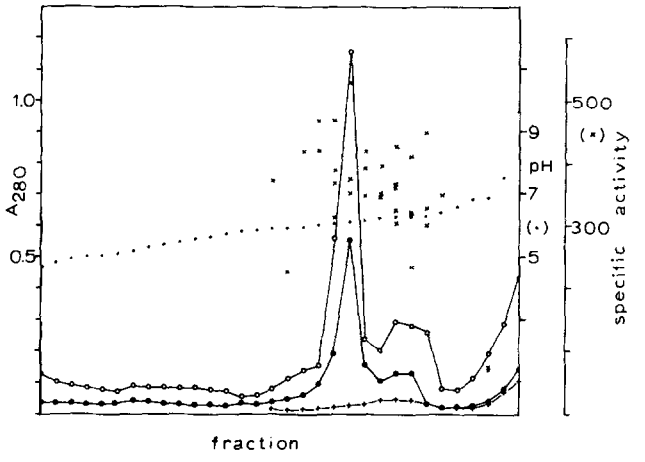

Figure 1. Typical isoelectric focusing pattern of bakers yeast enolase A and results of refocusing main and secondary species. 44 mg of enolase A was focused 44 hours at 4° on a column prepared with pH 5-7 ampholyte (open circles). The peak fraction from three such experiments (18 mg) was dialyzed and refocused (closed circles); the secondary peak fraction from three such experiments (6 mg) was also dialyzed and refocused (+). The x's in the figure are specific activities of fractions from these experiments (right ordinate).

made no difference. Evidently the magnesium in the enzyme is stripped from it during electrofocusing: measurement of the effect of successive additions of 5 mM EDTA and 10 mM magnesium chloride on the 296 nm absorbance of a sample of main peak (pI = 6.1) fraction showed the protein was metal-free (9).

Refocusing the major peak fraction from these two focusings for 45 hours again gave the same profile. The two main peak fractions from the refocusing were focused again, this time for 92 hours. This gave the same elution profile, although the amount of secondary and pH 7 material increased, relative to the major peak. In all cases, the specific activities of the main and secondary fractions were equal to that of unfocused enzyme (1).

The percentage recoveries of enzyme as the primary (pI = 6.1) peak were calculated. These values are plotted as functions of the amount of enzyme originally applied to the electrofocusing column (43-46 hour focusing values only) in Figure 2, upper graph. The recovery values obtained for different amounts of protein applied were then corrected to the values theoretically obtainable if 44 mg of protein were focused in each experiment, and these

corrected values are plotted as functions of focusing time in the lower graph of Figure 2.

The percentage recovery as pI = 6.1 enzyme increases with increasing protein focused, and decreases with increasing focusing time. The relative amount of secondary peak enzyme also appears to increase with electrofocusing time and with decreasing amounts of protein electrofocused (not shown), but this is harder to quantitate. Neither the presence or absence of magnesium prior to focusing, nor storage up to two weeks without magnesium at pH 6.1 in ampholyte-sucrose, nor refocusing per se had any effect on the relative amounts of primary and secondary material, or on the specific activity or isoelectric point of the primary species.

DISCUSSION

The data show the TEAE-chromatographed enzyme is inherently highly homogeneous, with an isoelectric point of 6.1, corresponding to the isoelectric point of the apoprotein. The secondary and more alkaline species observed are clearly produced from the primary species though the mechanism of this is less clear.

The secondary and more alkaline species cannot be deamidation products of the main species, as this would produce enzyme with lower isoelectric points. Nor are they likely to be due to the presence of one or two moles of bound magnesium. Prior addition of metal had no effect, and prolonged electrofocusing did not reduce the amount of more alkaline pH-focusing material but rather the reverse.

Their amounts appear to be partly a function of total electrofocusing time and also inversely proportional to amount of protein focused. These factors both suggest or are consistent with dissociation of the enzyme as a possible mechanism for production of the species with more alkaline isoelectric points. Removal of magnesium at more alkaline pH's leads to dissociation (10), and this may cause a conformational change in the subunits leading to eventual loss of activity. It is known that prolonged dissociating

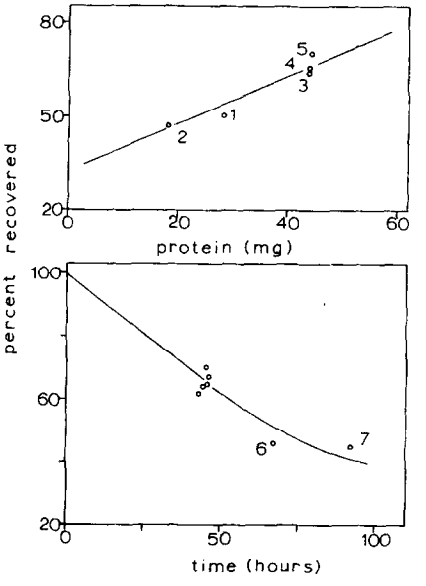

Figure 2. Percentage of applied enzyme recovered as primary (pI = 6.1) peak as functions of amount initially electrofocused (upper graph) or electrofocusing time (lower). The values in the upper graph were obtained after focusing 43-46 hours; those plotted in the lower graph were corrected to a common initial amount of 44 mg of protein applied. The conditions in each case were: 1) 28 mg of Mg-enzyme; 2) 18 mg of Mg-enzyme, major peak refocused; 3) 44 mg Mg-enzyme; 4) 44 mg enzyme, excess (20 μmoles) magnesium added beforehand; 5) 44 mg apoenzyme (deionized); 6) 44 mg Mg-enzyme; 7) 33 mg apoenzyme, second refocusing.

conditions destabilize the enzyme. It is also known that dissociation increases with pH (11).

We suggest that under electrofocusing conditions, a small fraction of pI = 6.1 apoprotein is constantly exposed to somewhat more alkaline pH's due to diffusion into regions of higher pH. At these higher pH's, this enzyme would dissociate to a greater extent, and some fraction of the subunits would alter in conformation to one with a more alkaline pI (6.3). This would in turn change to another conformation(s) with a yet more alkaline pI(s) which would probably eventually become inactive.

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