THE SUBUNIT STRUCTURE OF HUMAN PLACENTAL 17β-ESTRADIOL DEHYDROGENASE[1,2]

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Summary

Human placental 17β-estradiol dehydrogenase gave a single band by sodium dodecyl sulfate - polyacrylamide gel electrophoresis, corresponding to a molecular weight of approximately 33,500 daltons. Alanine was the only amino acid detected by N-terminal analysis. Ultracentrifugation gave a molecular weight of 67,700 daltons which suggests that in solution the enzyme exists as a dimer.

Introduction

A variety of molecular weights have been reported for purified human placental 17β-estradiol dehydrogenase. A value of 48,000 daltons (1) was obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Jarabak and Sack (2) obtained values of 62,000-

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65,000 daltons using gel filtration and cofactor binding techniques, whereas Descomps et al. (3) obtained 92,000 daltons by gel filtration.

We have re-examined the subunit nature of estradiol dehydrogenase using SDS-gel electrophoresis. The results together with N-terminal analysis provide strong evidence that the enzyme subunit is a polypeptide weighing approximately 33,500 daltons. Further, ultracentrifugal determination of the molecular weight of the enzyme in buffer containing 20% glycerol gives a value of 67,700 daltons, suggesting that under these conditions estradiol dehydrogenase exists as a dimer of possibly identical subunits.

Methods and Results

Enzyme purification. Estradiol dehydrogenase was isolated and purified from human term placentae by a modification of the procedure described previously (1). The enzyme was homogeneous by analytical disc gel electrophoresis at pH 8.9, 6.9 and 4.3. The purified enzyme was stored in either 10mM or 100mM potassium phosphate buffer, pH 7.2, containing 20% glycerol, 7mM 2-mercapto-ethanol and 5mM EDTA (storage buffer) at 4°. Under these conditions enzyme activity is preserved for many weeks.

SDS-gel electrophoresis. Three procedures (4,5,6) for examining protein - SDS complexes by polyacrylamide gel electrophoresis were used. All gels were stained with Coomassie Blue according to Fairbanks et al. (5).

Standard proteins were obtained from commercial sources and all chemicals were reagent grade. Acrylamide, N,N'-methylene bisacrylamide, and SDS were recrystallized before use.

(i) Initial experiments were performed using the procedure of Weber and Osborn (4) which employs 10% acrylamide gels. The system is uniform with respect to buffer (pH 7.0) and SDS (0.1%) concentrations. The enzyme gave a single band migrating with a mobility correponding to a molecular weight of 33,500 ± 1,000 daltons (Fig. 1). Identical results were obtained when estradiol dehydrogenase was treated prior to electrophoresis either by

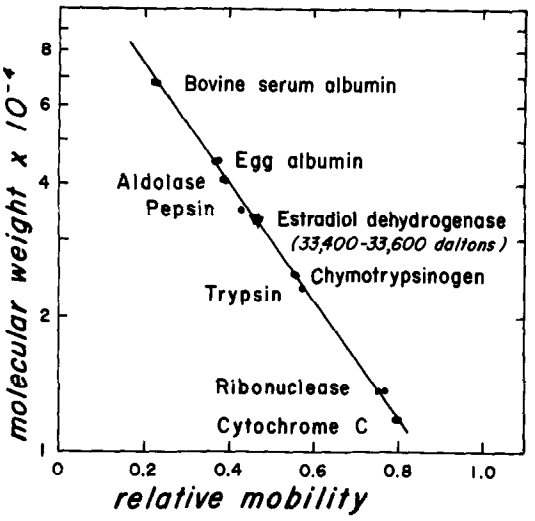

Fig. 1. Determination of the molecular weight of estradiol dehydrogenase by SDS-polyacrylamide gel electrophoresis (4). The relative electrophoretic mobilities are plotted against the logarithms of molecular weights of proteins of known size.

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carboxymethylation or by warming in 8M urea containing 1% SDS and 1% 2-mercaptoethanol either at 37° for two hours, for at 100° for five minutes.

(ii) Fairbanks et al. (5) have developed a procedure which utilizes 1% SDS during both pretreatment and electrophoresis. The gels are 5.6% acrylamide and the gel and electrophoresis buffers are pH 7.4; the sample is buffered at pH 8. When estradiol dehydrogenase was examined by this method a single band was obtained with a molecular weight of 33,700 ± 1,000 daltons.

(iii) The consistency with which a molecular weight of approximately 33,500 daltons was obtained under conditions which favor dissociation of protein subunits suggested that this is the limiting size of the polypeptide chain. However, this conclusion is in conflict with a previous publication from this laboratory (1) which reported that a single band with a molecular weight of 48,000 daltons was obtained by SDS-gel electrophoresis of an enzyme preparation of similar specific activity. Since we found no evidence for polypeptides with molecular weights less than 33,500 daltons it did not appear as if the 48,000 dalton unit could be a complex of a 33,500 dalton

subunit and one or more polypeptides of smaller molecular weight. Accordingly, we repeated the determination of molecular weight by the technique previously employed.

In this method (6) proteins are pretreated with 0.1% SDS and subjected to electrophoresis at pH 8.9 in gels (7.5% or 9.4% acrylamide) containing 0.025% SDS. The electrophoresis buffer (pH 8.3) does not contain SDS. In 7.5% gels the enzyme migrated slightly more slowly than egg albumin (M.W. 45,000), and faster than bovine serum albumin (M.W. 68,000) in accordance with previous results (1). In 9.4% gels estradiol dehydrogenase migrated as a diffuse band between egg albumin and bovine serum albumin. The relatively slower migration observed for estradiol dehydrogenase in the gel system (iii) as compared to the systems discussed above ((i) and (ii)), and the diffuseness of the band found in the 9.4% gels suggest that the conditions are inadequate to give a homogeneous population of polypeptide chains complexed with uniform quatities of SDS (7). Moreover, poor resolution of proteins of molecular weight less than 45,000 daltons was obtained. The importance of a high concentration of SDS throughout gel electrophoresis is also emphasized by the observations of Kempe and Noltmann (8). We conclude therefore that the value of 48,000 daltons previously assigned to the polypeptide unit of estradiol dehydrogenase (1) is incorrect, and that the correct value is approximately 33,500 daltons.

Molecular weight determination by ultracentrifugation. The molecular weight of the enzyme in a storage buffer was determined using an ultracentrifugal technique that permits accurate determinations in any solvent (9). The technique involves conditions of initial high speed meniscus depletion followed by the attainment of low speed equilibrium. The amino acid composition of the enzyme (to be published) allowed a tentative value of 0.74ml/g to be assigned for the partial specific volume. This value was used in the molecular weight calculation. Duplicate samples gave identical results (Fig. 2) which correspond to a calculated molecular weight of 67,700 daltons.

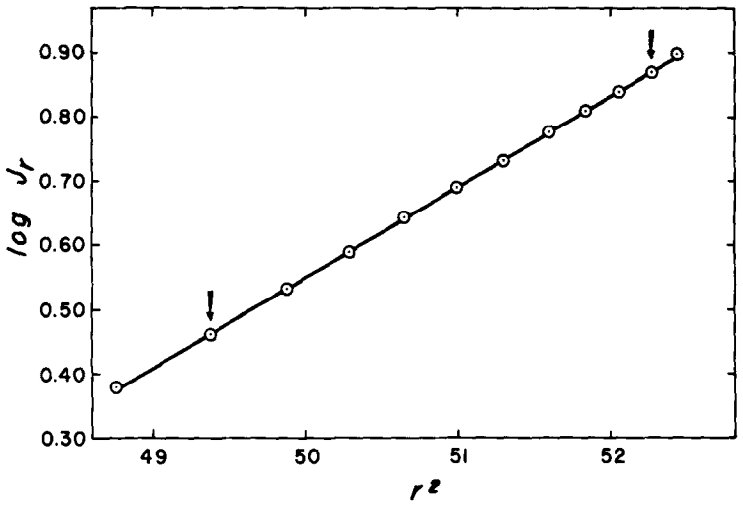

Fig. 2. Determination of the molecular weight of estradiol dehydrogenase by ultracentrifugation (8). Log J_r is plotted against r^2, where J_r is the fringe number at radial distance, r. A solution of estradiol dehydrogenase of approximately 1.7mg/ml was dialyzed to equilibrium for 70 hours at 4° against a 10mM potassium phosphate buffer pH 7.2, containing 20% glycerol, 7mM 2-mercaptoethanol and 5mM EDTA. Samples of the dialyzed enzyme were subjected to ultracentrifugation at 20°C and monitored by time-lapse photography. The dialysis buffer was used as the blank solution. Buffer density was 1.0610g/ml, $\bar{v}$, 0.74ml/g, and equilibrium speed was 9945 rpm.

These results suggest that in 20% glycerol, estradiol dehydrogenase exists as a dimer.

N-terminal analysis. This was performed by the [^{14}C]-fluorodinitrobenzene procedure of Allen (10). 1% Trimethylamine was used instead of $NaHCO_3$ to maintain alkalinity during the initial reaction. The [^{14}C]-dinitrophenyl-amino acids were located by radioautography after 2-dimensional thin-layer chromatography. After the thin-layer plate had been spread with a binding solution (11) the radioactive areas were cut out and the radioactivity determined in a scintillation counter.

Alanine was the only N-terminal amino acid detected when a 65µg sample of carboxymethylated estradiol dehydrogenase was analyzed. The yield of alanine based on a subunit molecular weight of about 33,000 daltons was 52%. The low yield of alanine is probably due, in part, to the instability of

substituted N-terminal amino acids during acid hydrolysis (12). The possibility that the enzyme has two different subunits is not entirely ruled out, since each could terminate in alanine, one could terminate in a blocked amino acid (e.g. N-acetylserine), or a proline residue the dinitrophenyl derivative of which would probably be destroyed during prolonged acid hydrolysis.

Discussion

A feature of the procedures that have resulted in purification of estradiol dehydrogenase to homogeneity has been the incorporation of glycerol in concentrations of 20% or higher in the buffers (1,3,13). Glycerol protects the enzyme against the rapid inactivation that occurs in aqueous buffers below 11° (14). We have determined a molecular weight of 67,700 daltons for estradiol dehydrogenase by ultracentrifugation at 20° in a buffer containing 20% glycerol. Under these conditions the enzyme is stable. This value may be slightly in error because the calculation of partial specific volume did not take into account the presence of glycerol in the solvent. This result, the N-terminal analysis, and the revised estimate of 33,500 daltons for the size of the polypeptide unit suggest that under the usual conditions of preparation the enzyme exists as a dimer. Our work is in agreement with the results of Jarabak and Sack (2) who estimated the molecular weight of the enzyme as 62,000-65,000 daltons by gel filtration and pyridine nucleotide binding studies. Gel filtration was carried out in an aqueous buffer at room temperature. Descomps *et al.* (3) obtained a value of 92,000 daltons by gel filtration at an unspecified temperature and in the presence of a substrate analogue. Estradiol and some substrate analogues protect the enzyme against cold inactivation although to a lesser extent than does glycerol. It seems possible that the higher estimate of molecular weight might be attributable to a modification in the physical nature of estradiol dehydrogenase, perhaps to a trimeric state, caused by the presence of the substrate analogue. The existence of a concentration

dependent monomer-dimer-trimer association has been proposed for the enzyme by Hagerman (15), who used an impure enzyme fraction prepared in buffers lacking glycerol but containing estradiol. Clearly, useful information could be gained by applying the ultracentrifugal technique used in the present work to an examination of the physical state of estradiol dehydrogenase in solution under a variety of conditions.

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