SUBUNIT COMPOSITION OF HORSE LIVER ALCOHOL DEHYDROGENASE ISOENZYMES*

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1. Introduction

During the last few years several protomeric enzymes have been shown to exist in multiple molecular forms produced by random combination of non-identical subunits. Three isoenzymes of alcohol dehydrogenase (ADH; E.C. 1.1.1.1) are found in rhesus monkey and man [1–3] indicating that ADH is a dimer composed of two different subunits. Recently a dimeric structure has been reported for the main fraction of alcohol dehydrogenase from horse liver [4,5]. However, in this species more than three enzymatically active fractions can be distinguished by electrophoresis and chromatography [6,7]. Starch gel electrophoresis reveals up to nine fractions [8,9]. The present communication reports on the subunit composition of these horse liver ADH fractions.

2. Experimental

2.1. *Electrophoresis*

The method for the agar gel electrophoresis has been described previously [1]. Starch gels were prepared according to Smithies [10]; gel buffer, Tris-HCl 0.025 M, pH 8.5; vessel buffer, Tris-HCl 0.3 M, pH 8.5. The electrophoresis was carried out with 10 V/cm during 5 hr at 4°C. ADH bands were visualized by activity staining [3].

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2.2. *Monomerization and hybridization*

Reversible dissociation was performed under conditions described by Drum et al. [11]. Incubation of ADH with 8 M urea and 0.1 M mercaptoethanol in 0.1 M sodium phosphate buffer pH 7.5 at 21°C led to a complete loss of activity within 10 min. The activity could partially be restored by dilution with 10 volumes of the same buffer containing 6×10^{-6} M zinc chloride and 6×10^{-4} M NADH and subsequent incubation for 2 hr at 21°C. The reactivation mixture was dialyzed against 100 volumes of 0.1 M sodium phosphate buffer pH 7.5 for 14 hr at 4°C prior to activity measurements and electrophoresis.

3. Results and discussion

As shown in fig. 1, the electrophoresis of crude horse liver extracts on agar gel reveals five bands as previously reported [2,6,12,13]. The higher resolution provided by starch gel makes it possible to demonstrate that the broad band II on agar gel consists of three distinct subbands (IIa, IIb and IIc). They can be separated on a preparative scale by chromatography on DEAE cellulose. Starch gel electrophoresis of crude liver extracts reveals two additional bands between isoenzyme III and IV which we are naming IVa and IVb. Similar electrophoretic patterns showing up to nine fractions have recently been described by Pietruszko et al. [8].

Based on kinetic studies of substrate specificity or effects of inhibitors and some hybridization experiments we have suggested a subunit composition AA, AB and BB for isoenzyme III, IV and V respectively [7]. The results of monomerization and reactivation

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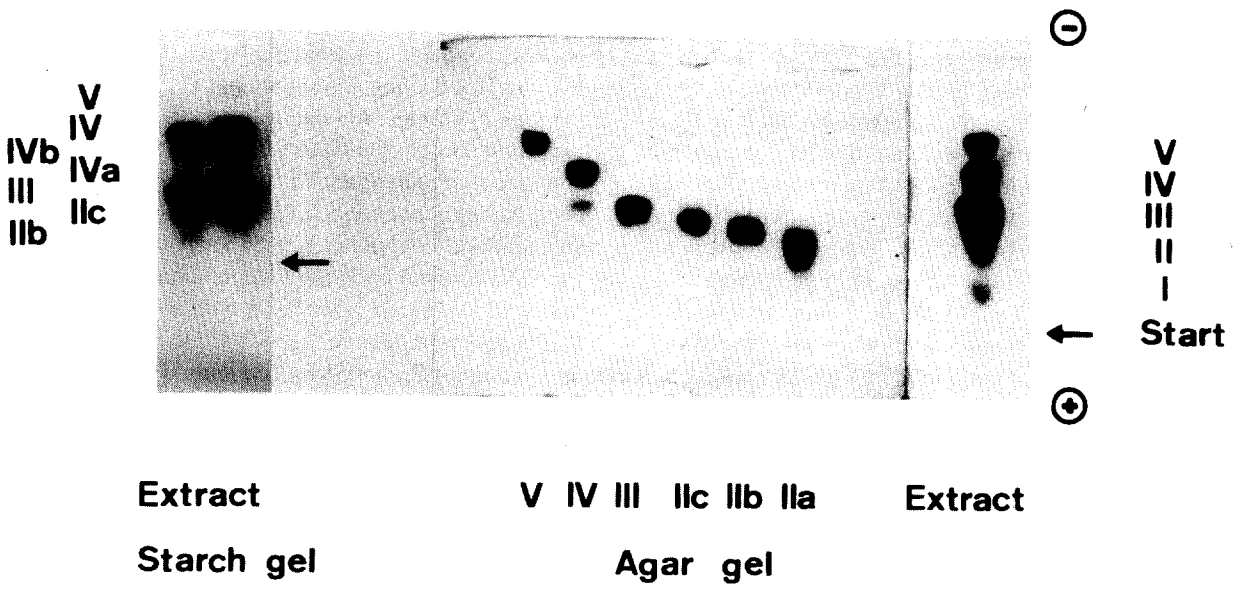

Fig. 1. Electrophoresis of horse liver ADH. Electrophoretic pattern of crude extract on starch gel at pH 8.5, Tris-HCl buffer (left) and on agar gel at pH 9.0, Tris-HCl buffer (right). Electrophoresis on agar gel at pH 9.0, Tris-HCl buffer of fractions separated and purified on ion exchange celluloses (middle). ADH bands are visualized by activity staining.

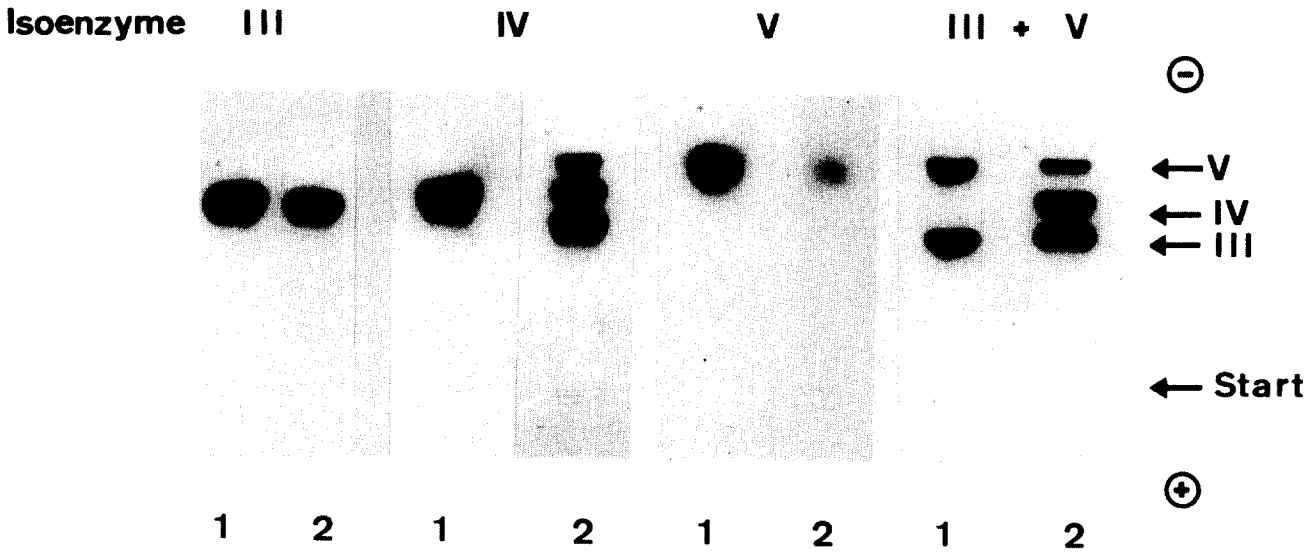

Fig. 2. Hybridization of horse liver ADH isoenzymes. Electrophoresis on agar gel at pH 9.0, Tris-HCl buffer was carried out before monomerization [1] and after reactivation [2]. For experimental procedure see text.

of pure isoenzyme III, IV and V and of the hybridization of a mixture of III and V are shown in fig. 2. As expected, isoenzyme III and V behave like enzymes containing identical subunits. The high degree of monomerization is exemplified by the extensive formation of the hybrid enzyme during reversible inactivation of a mixture of isoenzyme III and V. In agreement with these findings the hybrid enzyme IV yields all three isoenzymes upon monomerization and subsequent recombination. None of the other electrophoretic fractions known to exist in crude horse liver extracts are formed in any of these experiments. These results are in accordance with the observations recently published by Pietruszko and Theorell [14].

Crystals of fractions IIc and III obtained from 5% (v/v) methanol in phosphate buffer are shown in fig. 3. If the repeatedly washed crystals of isoenzyme III are redissolved and submitted to electrophoresis, pure isoenzyme III is found. In the supernatant, however, isoenzyme III partly converts to fraction IIc (fig. 3a); the amount of IIc increases with the time of storage. An analogous conversion of fraction IIc to isoenzyme III can be observed (fig. 3b). The conversion of isoenzyme III to fraction IIc is a slow process, requiring weeks for the formation of considerable amounts of IIc. The rate of conversion is not enhanced under the conditions of monomerization and reactivation. On the other hand, the reverse process, that is the conversion of fraction IIc to isoenzyme III, occurs at a faster rate and is accelerated by monomerization. As shown in fig. 4a, isoenzyme III is formed to a large extent during the 20 hr required for monomerization, reactivation and dialysis of fraction IIc. Isoen-

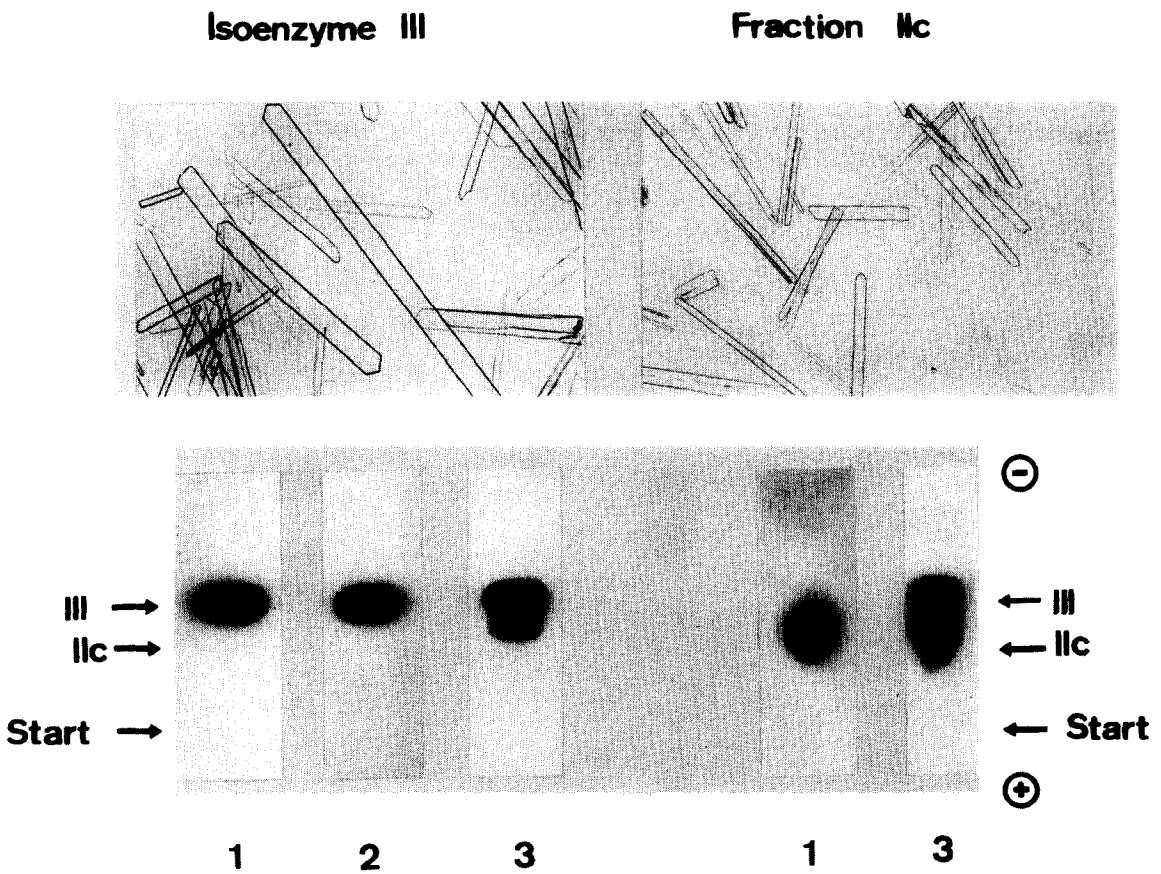

Fig. 3. Crystallization of horse liver ADH. a: Isoenzyme III; b: Fraction IIc. Crystals were collected from 5% (v/v) methanol in phosphate buffer. Electrophoresis on starch gel at pH 8.5, Tris-HCl buffer: (1) chromatographically purified fraction; (2) redissolved crystals; (3) supernatant.

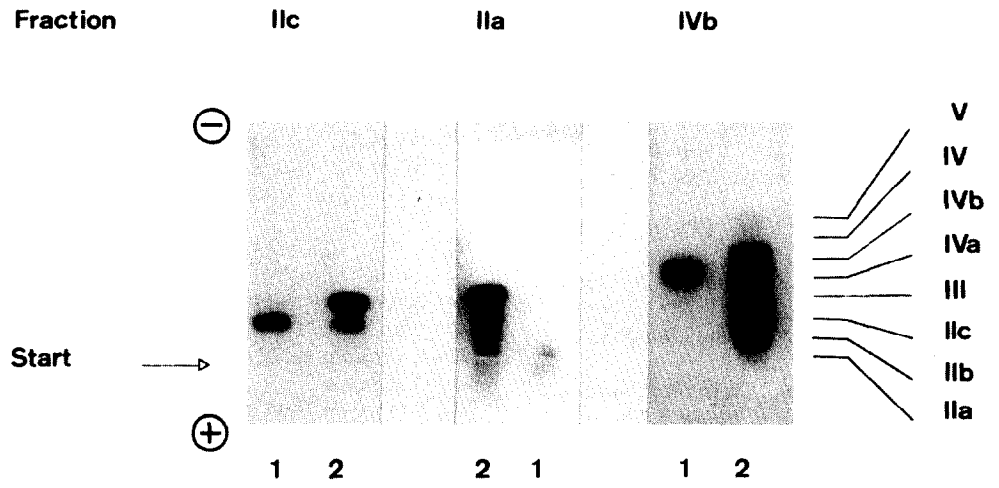

Fig. 4. Monomerization and reactivation of horse liver ADH. a: Fraction IIc; b: Fraction IIa; c: Fraction IVb. Electrophoresis on starch gel at pH 8.5, Tris-HCl buffer: (1) before monomerization and (2) after reactivation.

zyme III, upon standing in solution, forms fractions IIb and IIa in addition to fraction IIc, indicating a further interrelation between these fractions. In agreement with this observation monomerization and reactivation of fraction IIa yields a mixture of fractions IIa, IIb, IIc and isoenzyme III (fig. 4b). The existence of more than three isoenzymes (III, IV and V) could be explained in several ways:

(1) By the assumption of further subunits differing in primary structure from subunit A and B.

(2) By processes involving the cleavage of covalent bonds (disulfide bridge, peptide or acid amide). The full interconvertibility between the fractions IIa, IIb, IIc and isoenzyme III, however, excludes these two possibilities.

(3) The changes in charge underlying the differences in electrophoretic mobility and chromatographic properties of these interconvertible fractions could result from a reversible attachment of ligands as reported for drosophila ADH [15]. Since the interconversion occurs in the absence of free ligands such as zinc and coenzyme, this possibility seems unlikely. Furthermore, identical electrophoretic patterns were obtained in presence or absence of zinc, EDTA or coenzyme during monomerization and/or reactivation.

The concept of conformers advanced by Kaplan [16] offers an alternative explanation. The polypeptide of subunit A could adopt more than one conformation thus leading to subunits with the same primary structure but differing in physical-chemical properties. The existence of one alternate conformation (A′) would give rise to two conformers (AA′ and A′A′). Since there are more than two fractions interconverting with isoenzyme III (IIc, b and a) two conformationally different subunits (A′ and A″) are suggested. The question arises whether A′ and/or A″ can form enzymatically active dimers with the B subunit. As shown in fig. 4c, the reversible inactivation of the fraction IVb yields isoenzyme V, IV and III as well as the fractions IVb, IVa, IIc, IIb and IIa. This finding suggests that the fraction IVb represents a hybrid dimer between one of the conformationally changed A subunits and B.

The results show two different processes to be responsible for the multiple molecular forms of horse liver alcohol dehydrogenase:

(1) Isoenzymes III, IV and V are formed by random combination of two subunits A and B differing in primary structure.

(2) Three conformations of the A polypeptide (A, A′ and A″) seem to exist. They are sufficiently stable thermodynamically to form catalytically active dimers *in vitro* as well as in the liver cell. A study of the primary structure of the subunits of these conformers is in progress in order to corroborate this hypothesis.

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