MAPPING STRUCTURAL PERTURBATIONS IN *ESCHERICHIA COLI* ASPARTATE TRANSCARBAMYLASE BY MEDIUM RESOLUTION HYDROGEN EXCHANGE

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Allosteric regulation of *Escherichia coli* aspartate transcarbamylase depends upon ligand-induced changes in subunit interactions. Crystallographic studies of the unliganded enzyme (1) and the CTP (1), ATP(2), and PALA (3) complexes have now been carried to 3 Å resolution. The thermodynamics of ligand binding and assembly have also been examined by reaction microcalorimetry, differential scanning calorimetry, potentiometry, and auxiliary methods (4–7). The availability of both structural and thermodynamic information makes it possible to begin to ask what structural elements are involved in transmitting signals between binding sites.

Both assembly ($2c_3 + 3r_2 \rightarrow c_6r_6$) and binding of ligands to c_3 and r_2 produce substantial changes in net rates of exchange (8). Studies at increased resolution should therefore identify regions whose energetics are perturbed by interactions. Nuclear magnetic resonance (NMR) and neutron diffraction studies of individual protons are precluded because the subunits are too large and the crystal structures are not known. However, the medium resolution method developed by Rosa and Richards (9) and Englander and his colleagues (10) can be applied.

At pH 3 and 0°C the half-time for exchange is $>1\frac{1}{2}$ h while peptides can separate by reverse phase HPLC in <1 h. Hence, the protein can be cleaved with acid proteases at various times during exchange and the isotopic content of individual peptides determined. After corrections for limited exchange during analysis have been applied, exchange-out curves for individual peptides can be generated.

MATERIALS AND METHODS

r_2 was prepared, characterized by standard methods (7) and used within two weeks. ^{3}HOH was added to protein equilibrated with the experimental buffer to give a final specific activity of 100 mC/ml. After equilibrium, exchange-out was initiated by passing the sample through a G-25 Sephadex column (1 × 14 cm) and continued in a rapid dialysis apparatus. At suitable times 0. 4 ml aliquots were withdrawn, equilibrated with 100 mM Na-malonate, pH 3.0 by passage through a second column, frozen in dry-ice–acetone and stored at −70°C.

For analysis, samples were quickly melted (at 0°C) and pepsin added to a final concentration of 300 μg/ml. After 5 min digestion, the sample was applied to a Waters μBondapak C-18 reverse phase column (Waters Associates, Inc., Milford, MA) equilibrated with 50 mM NaH_2PO_4, pH 2. 8 and eluted with a 3%/min dioxane-MeCN-NaH_2PO_4 gradient. Peptides were collected and OD_{220} and ^{3}H cpm determined. Corrections for background and exchange during analysis were made with the methods described in reference 10.

Peptides were identified by precolumn OPA derivation of their constituent amino acids and separated on a reverse phase Rainin C-18 column. Hydrolysis was performed in 4N CH_3SO_3H + 0. 2% 3-(2-ethylamino)indole at 110°C 25 μ pressure for 24 h.

RESULTS

Fig. 1 shows elution profiles for unliganded r_2 in which both exchange-in and exchange-out were carried out at pH 7, 0°C (exchange in time: 20 h). Eight of the peptides that were separated were identified by amino acid analysis; several identifications have been confirmed by NH_2-terminal analysis. The peptides identified correspond to ~50% of the sequence. Peptides corresponding to residues 20–60 have not been identified, probably because this region is rich in leucine and yields small fragments. Although the radioactive background is significant, seven peptides have ^{3}H contents well above background. Semilog plots of these data and comparable data obtained in the presence of 2. 5 mM ATP are shown in Fig. 2. Measurements of net rates

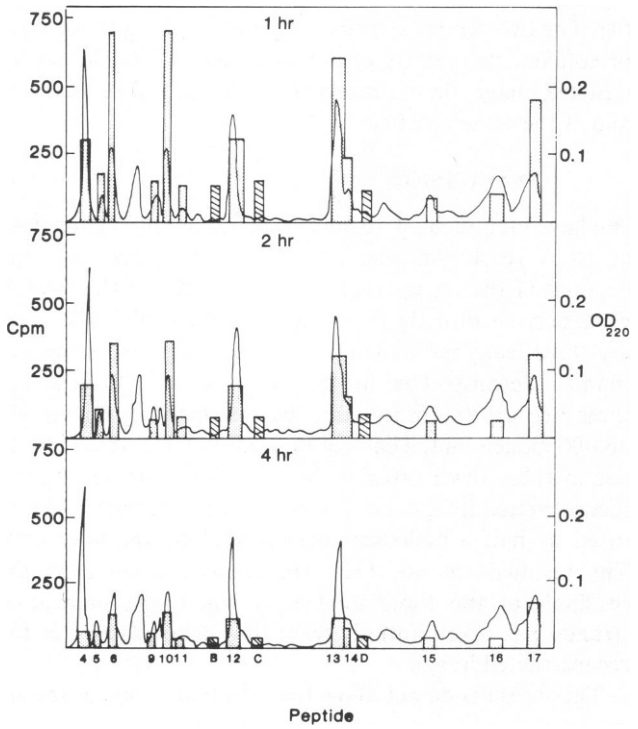

FIGURE 1 HPLC elution profile of r_2 after 1, 2, and 4 h exchange at pH7, 0°C. Smooth curve: OD_{220}; bar graphs: cpm/100μl.

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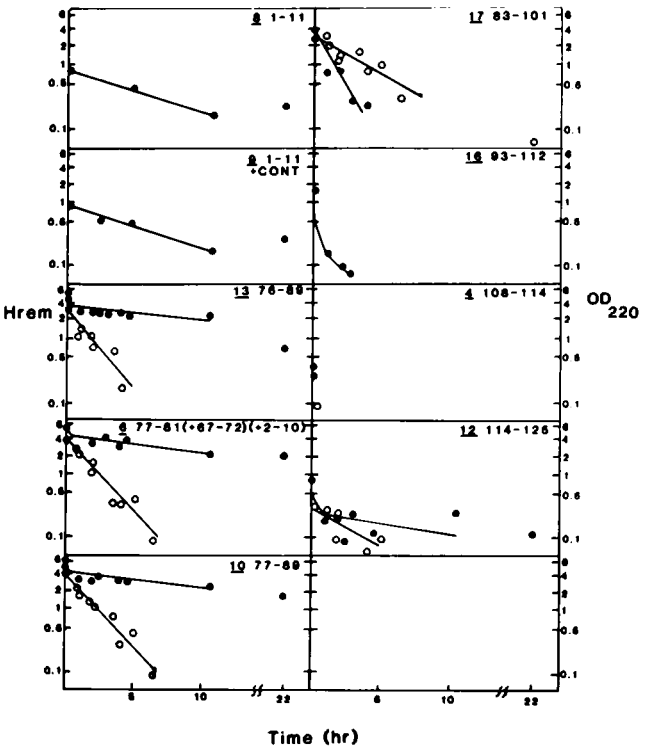

FIGURE 2 Effect at ATP (2. 4 mM) on exchange rates of r_2 peptides (pH 7, 0°C). ○, −ATP; ●, +ATP.

of exchange indicate that the exchange of ~70 protons can be followed under these conditions and that ATP reduces the net rate of exchange fourfold. Sixteen of these protons occur in peptides which have been identified. ATP generally slows exchange, but accelerates exchange in peptide 83–101 by a factor of three.

A second experiment carried out at pH 8. 5 and 32°C allowed a second kinetic class to be examined. Protons examined under these conditions exchange ~1,000 times slower than those in the first experiment. Measurements of net rates of exchange indicate that there are 20 protons in this class, and that their average rate of exchange is not changed significantly by ATP. Control experiments show that K_d is ~0.5 mM and that the protein does not denature under these conditions. In the medium resolution experiment, it was found that six protons in peptides 1–11 and 77–89 were labeled and that their rates of exchange were essentially the same with and without ATP.

DISCUSSION

Locations of the peptides in the three-dimensional structure are given in Table I. The three peptides which show the most clear-cut effects (1–11, 77–89, 83–101) lie in the nucleotide binding domain. Although it is not surprising that protons near the nucleotide binding site are perturbed when nucleotides bind, it is noteworthy that while some rates decrease, others increase, perhaps as a result of strain. The most slowly exchanging protons examined are unaffected; they are apparently part of very stable structures whose stablility does not change appreciably when ATP binds. It is also of interest that some protons in the amino-terminal fragment exchange quite slowly, because this region appears unstructured in the crystal.

TABLE I
IDENTITY AND LOCATION OF PEPTIDES

Peptide	Residues	Secondary Structure	Domain
4	108–114	None	Zinc
8	1–11	None	Nucleotide
9	1–11 + Contaminants	None	Nucleotide
10	78–89	End of H2, S4	Nucleotide
12	114–125	Beginning of S7	Zinc
13	77–89	End of H2, S4	Nucleotide
16	93–112	End of S5, S6	Nucleotide + Zinc
17	83–101	S4, Turn, S5	Nucleotide

*Symbols defined in reference 1.

It will be of interest in the future to study the effects of assembly on these peptides because they are far from the c:r contact. It will also be important to study the effects on the zinc domain of both ligand binding and assembly. This will require finding conditions that label peptides produced from this domain by pepsin, and also using proteases with different specificities to obtain different fragments.

The principal limitation of this method at present is the separation with columns now available. The power of the method will increase as better columns become available.

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CONFORMATIONAL DRIFT OF LACTATE DEHYDROGENASE

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The loss and recovery of the enzymic activity of lactate dehydrogenase (LDH) subjected to pressure have been studied by Jaenicke and co-workers (1, 2). On the assumption that the fraction of enzyme activity after decompression accurately represents the degree of dissociation of the tetramer into monomers at the incubation pressure, they calculated a standard change in volume on dissociation of −500 ml/mol. They ascribe the slow reactivation at atmospheric pressure to the reassociation of the monomers. We have used fluorescence polarization methods (3) to monitor the degree of dissociation of LDH under pressures of 1 atmosphere to 3 kbar. When degree of dissociation and enzymic activity are separately measured they reveal a more complex situation than that postulated by Jaenicke et al.

RESULTS

The product of the dissociation under pressure is shown to be the monomer by its 30 ns rotational relaxation time and by the uniform appearance in time of the three mixed isozymes when the M_4 and H_4 lactate dehydrogenases are jointly subjected to high pressure (4). Fig 1 shows the degree of dissociation, calculated from the tryptophan fluorescence polarization, observed at pressures of up to 2.5 kbar, at three protein concentrations. The apparent change in volume upon dissociation is about −300 ml/mol. The initial polarization is recovered "immediately" after decompression, indicating prompt reassociation of the monomers to form an aggregate of the volume of original tetramer. This is confirmed by observations of electrophoretic mobility and rotational relaxation time of the decompressed preparations. However, these newly associated tetramers have both diminished catalytic activity (1, 4) and subunit affinity (4). The original enzyme activity gradually returns after a time that increases with the magnitude of the applied pressure and the time of pressure incubation. From the above information we conclude that the dissociated monomers undergo a progressive loss in conformation, i.e., a "conformational drift," whose extent depends on both magnitude and time of pressure application, and that these monomers reassociate into inactive tetramers after decompression. Fig. 1 shows that measurable degrees of dissociation require pressures in excess of 1 kbar. However, appreciable amounts of inactivation, as well as hybridization of mixed M_4 and H_4 isoenzymes, are seen after long incubation (3–24 h) at pressures <1 kbar (1, 4). We conclude that in these conditions a microscopic cycle of associations and dissociations takes place, and that because of the very short lifetime of the monomer in these circumstances (5) a great many such cycles are necessary to produce drifted monomers that result in inactive tetramers. As pressure and degree of dissociation increase so does the lifetime of the monomer and a proportionally faster conformational drift follows. At 2 kbar the enzyme is

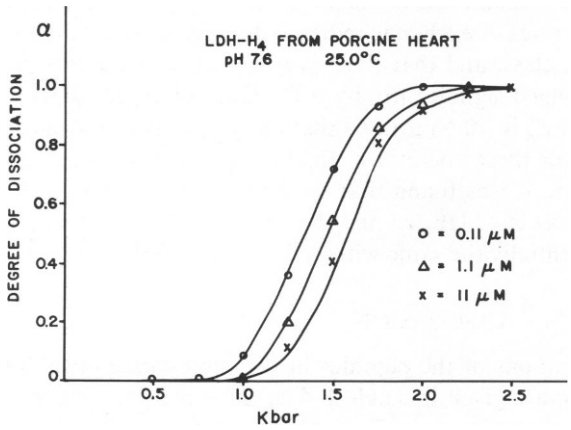

FIGURE 1 Plots of degree of dissociation from tryprophan polarization at three different protein concentrations against pressure, at 25°C. Solvent: 50 mM Tris-HCl with 1 mM EDTA and 1 mM DTE, pH 7.6. Excitation: 280 nm, through additional 7–54 Corning filter. Emission filter: WG320. Protein concentrations (micromolar): 0.11(o), 1.1 (Δ), 11 (×).

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