STRUCTURE, FUNCTION, AND POSSIBLE ORIGIN OF A BIFUNCTIONAL ALLOSTERIC ENZYME, *ESCHERICHIA COLI* ASPARTOKINASE I-HOMOSERINE DEHYDROGENASE I

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INTRODUCTION

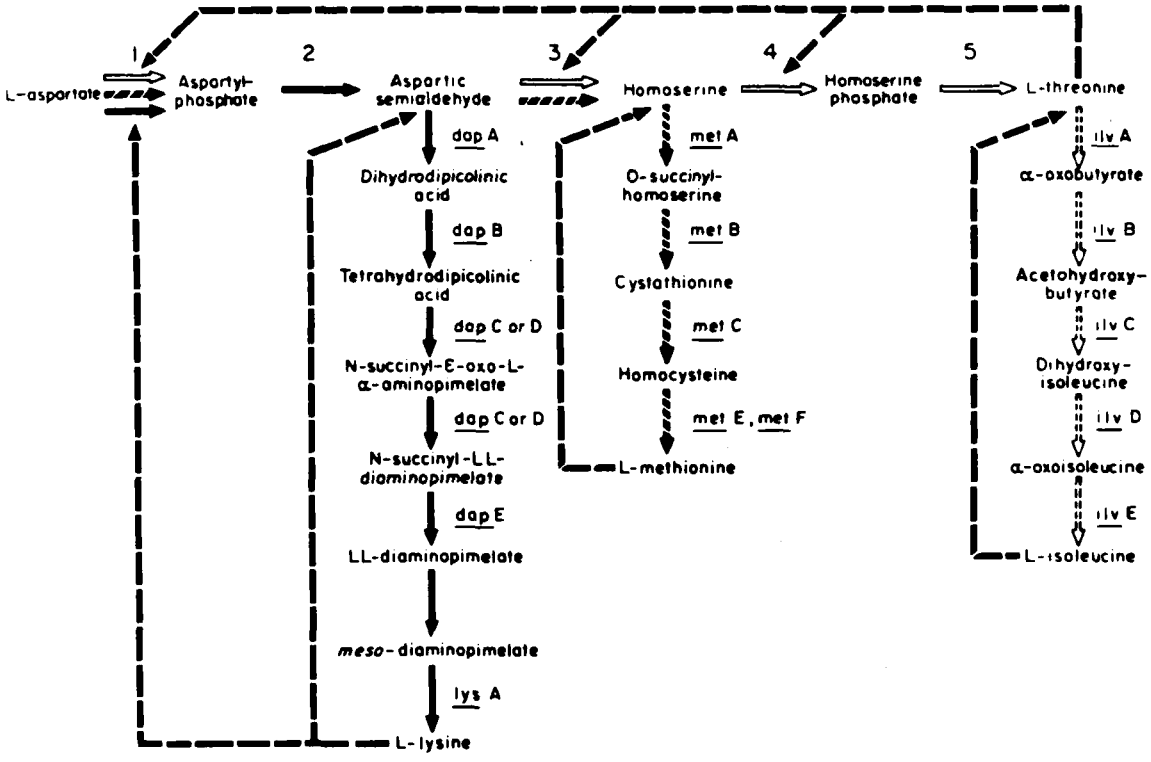
In *E. coli* K12, diaminopimelic acid and lysine, methionine, threonine, and isoleucine derive part or all of their carbon atoms from the four carbon atoms of aspartic acid. The corresponding pathway has been elucidated and is summarized below (Figure 1).

The synthesis of L-β-aspartyl-phosphate is catalyzed by three distinct aspartokinases, all of which catalyze the same reaction, but differ in the mode of regulation of their synthesis and activity. Aspartokinase I is inhibited by threonine and its synthesis is repressed by threonine plus isoleucine; the synthesis of aspartokinase II is repressed by methionine; lysine inhibits the activity and represses the synthesis of aspartokinase III. The reduction of L-aspartate semialdehyde to homoserine is likewise catalyzed by two distinct homoserine dehydrogenases. Homoserine dehydrogenase I synthesis is repressed by threonine and isoleucine, whereas methionine represses the synthesis of homoserine dehydrogenase II.

In the mid-sixties, it became apparent that aspartokinase I and homoserine dehydrogenase I activities are carried out by a single protein. Similarly, aspartokinase II and homoserine dehydrogenase II activities are carried out by another single protein.

The existence of three isofunctional aspartokinases and two isofunctional homoserine dehydrogenases allows an efficient and fine regulation of amino acid biosynthesis. Where there is an excess of a given end product, this causes the repression and the inhibition of one of the enzymes, and hence, of the amount of common intermediate synthesized. When the flow reaches the next branch point, this common intermediate, already in a lesser amount, does not take the direction of the end metabolite in excess, since there is a new enzyme subject to regulation at this point; it is directed toward the amino acid(s) which are needed for the growth of the organism. The whole biosynthetic machinery can be easily brought to a stop in a medium containing an excess of all the end products.

This review will deal with the chemical and immunological aspects of the bifunctional protein aspartokinase I-homoserine dehydrogenase I, which carries out the catalytic activities that are feedback-inhibited by L-threonine.



Reaction	Name of the enzyme	Abreviation	Name of the corresponding genes	Regulation of biosynthesis by:	Regulation of activity by:
1	Aspartokinase I	AK I	thr A [a]	thr and ile	thr
1	Aspartokinase II	AK II	met L [b]	met	0
1	Aspartokinase III	AK III	lys C	lys	lys
2	Aspartic semialdehyde dehydrogenase	ASADH	asd	lys, thr, met	0
3	Homoserine dehydrogenase I	HDH I	thr A [a]	thr and ile	thr
3	Homoserine dehydrogenase II	HDH II	met M [b]	met	0
4	Homoserine kinase	HSK	thr B	thr and ileu	thr
5	Threonine synthetase	TS	thr C	thr and ileu	n.d.

FIGURE 1. The biosynthetic pathway for lysine, methionine, threonine, and isoleucine. The dotted arrow interrupting the biosynthetic pathways indicates points of feedback inhibition. The broken arrows indicate repression by methionine; the dotted hollow arrows indicate trivalent repression by leucine, valine, and isoleucine. All the reactions shown with hollow arrows leading from aspartate to threonine, except the second, are subject to bivalent repression by threonine and isoleucine.

For a better understanding of the implications of the findings reported below, it is important to summarize the genetic organization of that part of the bacterial chromosome which is concerned more specifically with the synthesis of threonine, and which, in addition to aspartokinase I-homoserine dehydrogenase I, codes for homoserine kinase and threonine synthetase (respectively, Reactions 1 and 3, 4 and 5 of Figure 1). Figure 2 schematizes this genetic structure, now known as the threonine operon.

The *thrA* gene is organized into two cistrons, $thrA_1$ and $thrA_2$, coding respectively for one of the two activities of the bifunctional enzyme. This will be discussed in the body of the review.

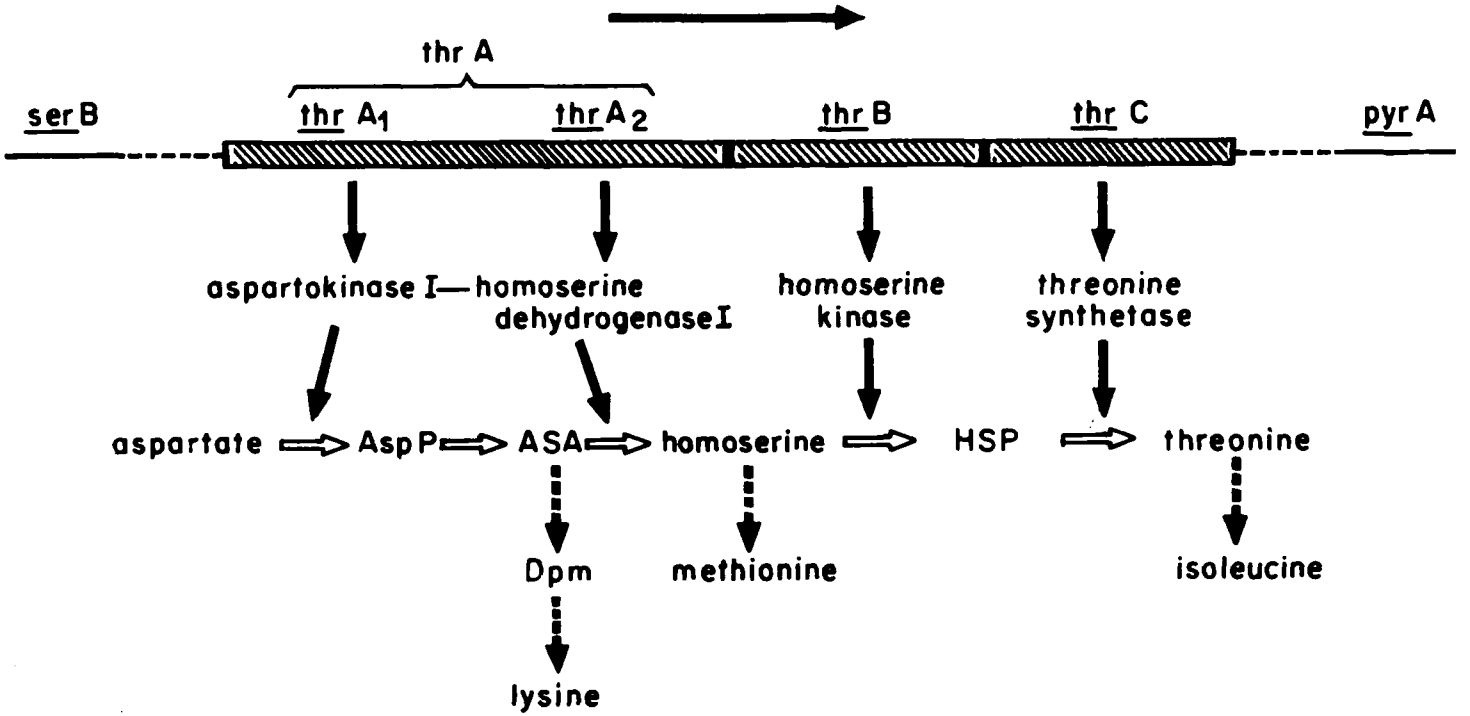
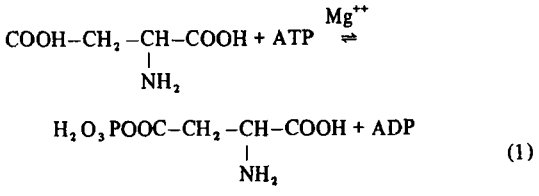


FIGURE 2. The threonine operon. AspP, ASA, Dpm, and HSP stand, respectively, for β-L-aspartyl-phosphate, L-aspartate semialdehyde, *meso*-diaminopimelate, and homoserine phosphate.

ASPARTOKINASE I-HOMOSERINE DEHYDROGENASE I

The Reactions Catalyzed

Aspartokinase I-homoserine dehydrogenase I has been obtained in the pure, homogeneous state by classical fractionation methods.[1,2] It catalyzes the two following reactions:



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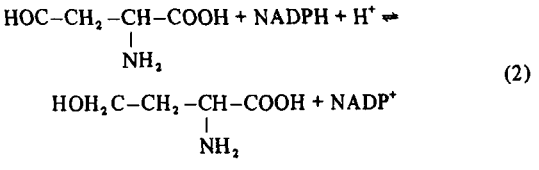


(2)

The conditions of assay, the enzyme units, have been repeatedly described.[3] Table 1 describes some of the kinetic parameters of the reactions.

Both reactions require K^+ ions for activity.[4,5] In addition, K^+ is important for the integrity of the quaternary structure, as will be discussed later. K^+ is known to be necessary for the activity of many kinases, but its requirement for a dehydrogenase is somewhat atypical.

Broussard et al.[6] have found that this requirement can be obviated by low concentrations of ATP-Mg. Even with ATP-Mg, no dehydrogenase activity is measurable in the total absence of K^+. Figure 3 shows that with 5 m*M* K^+, only 10% of the maximum activity is measurable; maximum activity can be reached with the addition of either 100 m*M* KCl or 50 μM ATP-Mg.[7]

Inhibition by L-Threonine and L-Serine

The inhibition of aspartokinase I[8] and homoserine dehydrogenase I[9] activities by L-threonine, the end product of the biosynthetic pathway, was recognized early. The inhibition of the kinase activity is competitive versus aspartate[8] and ATP-Mn.[10] The inhibition of the dehydrogenase activity is noncompetitive versus homoserine.[9]

The inhibition by threonine of both activities displays homotropic cooperative effects (Figure 4).[11,12] Whereas homotropic cooperative effects of the inhibitor without homotropic cooperative effects of the substrate are to be expected in V systems of the homoserine dehydrogenase type, they are not expected in K systems[13] of the aspartate transcarbamylase or the aspartokinase type. Such effects are not detectable with aspartokinase I, using aspartate as the substrate.[8] However, this result was obtained at a high K^+ concentration which, as we shall see below, favors the same active form (R-form) of the enzyme as aspartate. Therefore, Wampler and Westhead[5] have tested the aspartokinase at low K^+ concentrations; cooperative effects of aspartate

TABLE 1

Some Kinetic Parameters of the Reactions Catalyzed by Aspartokinase I-Homoserine Dehydrogenase I

K_m L-aspartate	= 1,500 μM
K_m ATP	= 180 μM
K_m L-aspartate semialdehyde	= 120 μM
K_m L-homoserine	= 6,500 μM
K_m $NADP^+$	= 110 μM
K_m NADPH	= 40 μM
Turnover number, aspartokinase	= 3,400 mol aspartyl phosphate synthesized × min^{-1} × $mol\ enzyme^{-1}$
Turnover number, homoserine dehydrogenase, forward	= 24,000 mol aspartate semialdehyde reduced × min^{-1} × $mol\ enzyme^{-1}$
reverse	= 2,000 mol homoserine oxidized × min^{-1} × $mol\ enzyme^{-1}$

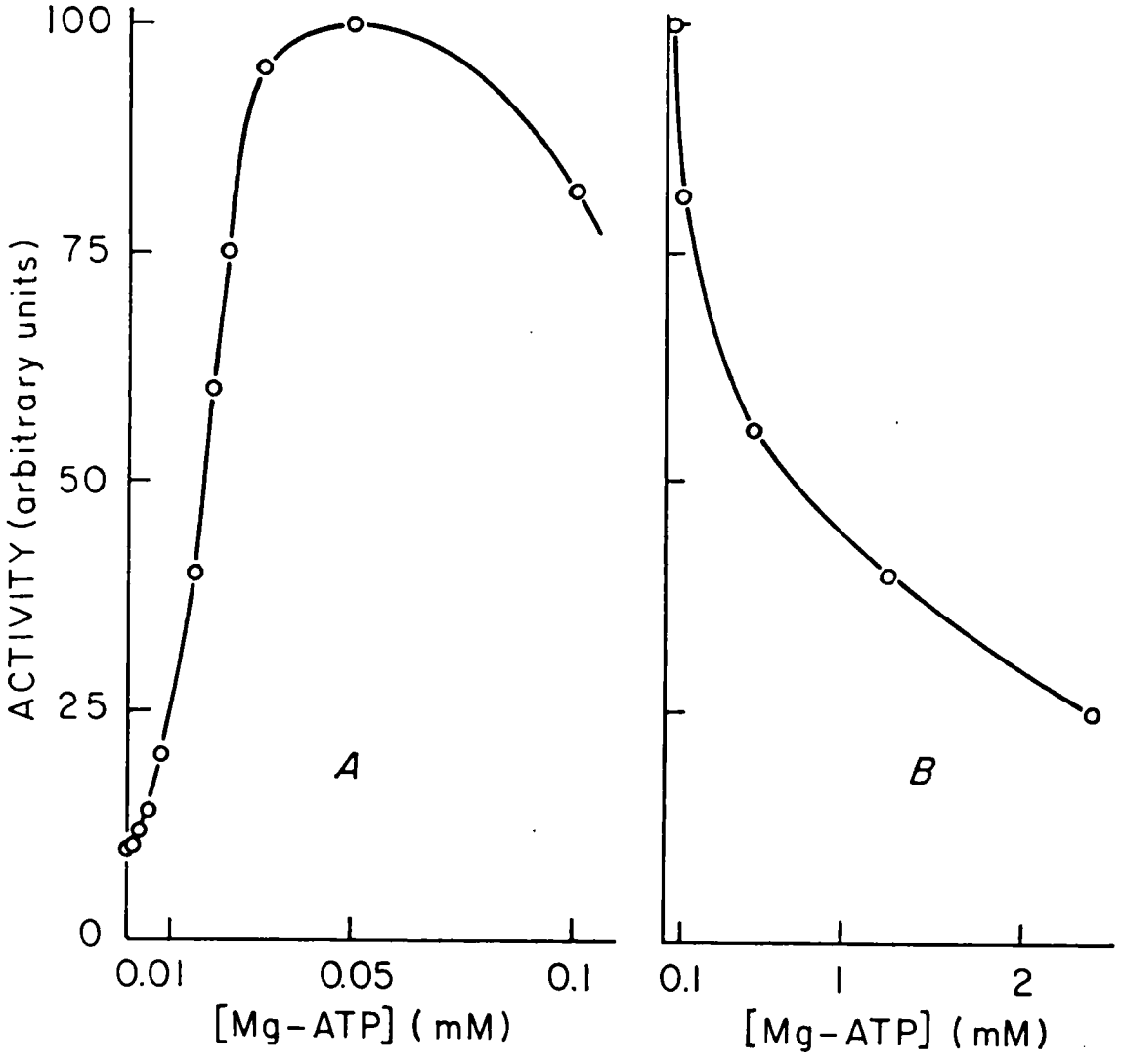


FIGURE 3. Effect of concentration of Mg-ATP on homoserine dehydrogenase activity, measured in the direction HS → ASA.

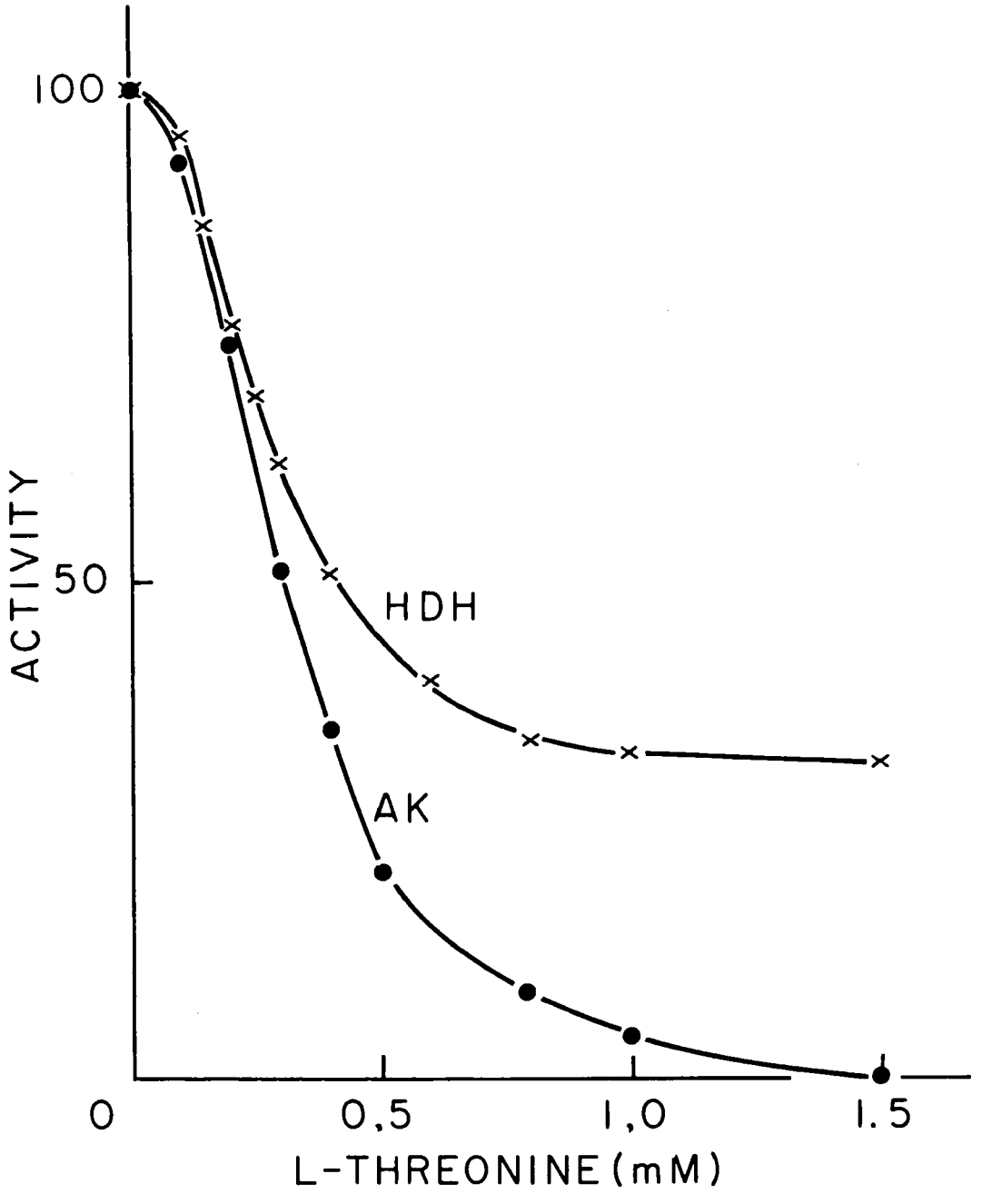


FIGURE 4. Homotropic cooperative effects of threonine on the two activities of aspartokinase I-homoserine dehydrogenase I.

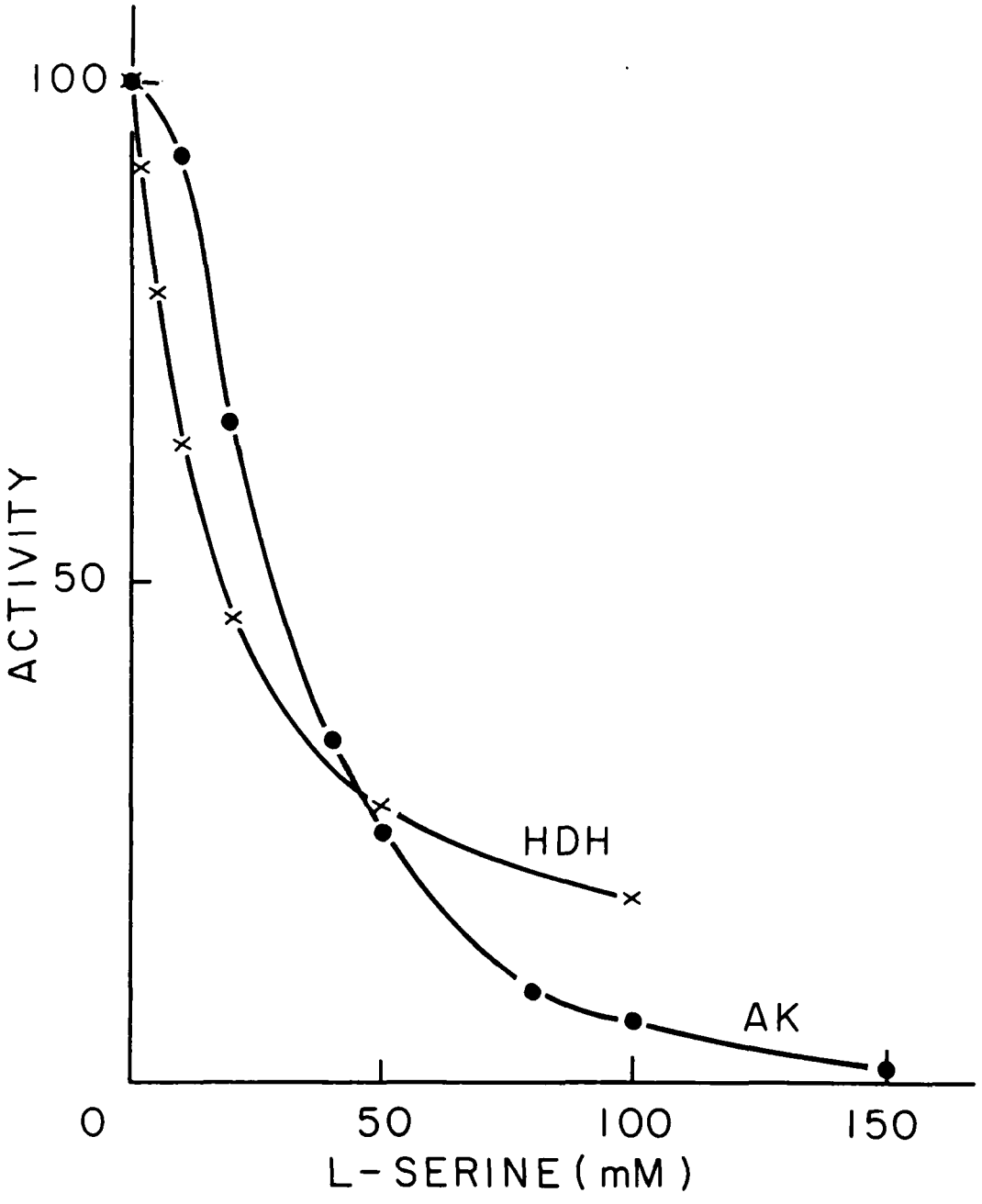


FIGURE 5. L-serine inhibits aspartokinase I cooperatively and homoserine dehydrogenase I noncooperatively.

were then observed. The same result was obtained by Patte[14] when the activity was measured at a high threonine concentration, where the inactive (T-form) is favored.

At low K^+ concentrations, threonine exerts its inhibition on homoserine dehydrogenase at lower concentrations, and the inhibition is of the noncooperative type.[7]

The inhibition by L-serine had been overlooked in the early work. It presents a considerable interest[15], since it displays cooperative effects on aspartokinase I, whereas it inhibits homoserine dehydrogenase I in a noncooperative manner (Figure 5). In addition, the inhibition by L-serine, in contrast to that exerted by L-threonine, is competitive with respect to homoserine.

Molecular Extinction Coefficient

The extinction coefficient of aspartokinase I-homoserine dehydrogenase I at 278 nm has been determined by quantitative analysis of the content in amino acids. A value of 54,000 ± 2,500 is obtained for a molecular weight of 86,000.

Molecular Weight

The molecular weight has been determined by hydrodynamic methods.[1] The value for the sedimentation coefficient obtained upon infinite dilution is $S_{20,w} = 11.5 \pm 0.2 \times 10^{-13}$ sec. This agrees very well with the results of sedimentation in a sucrose gradient using catalase and alcohol dehydrogenase as markers, where the peaks of both activities have an S value of $11.0 \pm 0.5 \times 10^{-13}$ sec.

Equilibrium sedimentation runs using interference optics were done. At the completion of the photography of the Rayleigh pattern, schlieren pictures were taken. This allowed the obtaining of values for M_w and M_z. Three concentrations of protein were used. The mean values found are:

$$M_w = 357{,}000 \pm 14{,}000; M_z = 350{,}000 \pm 30{,}000$$

Since M_z/M_w is not significantly different from unity, this is an additional confirmation of homogeneity. We shall give below the reasons for which we assume the molecular weight of the enzyme as 344,000.

Other workers have confirmed this value, also using hydrodynamic methods.[16] An independent measurement using light scattering has yielded a molecular weight value of 358,000 ± 35,000, in accord with the value determined by hydrodynamic methods.[17]

Subunit Structure

The reduced protein in solution in 6 *M* guanidinium hydrochloride dissociates to subunits of equivalent molecular weight.[18] A careful sedimentation equilibrium study, avoiding the pitfalls of the interpretations of results obtained in concentrated GuHCl solutions (i.e., taking into account the nonideal behaviour of the protein under these conditions), yields a value of approximately 80,000 for the molecular weight of the subunits.[16]

Values varying between 80,000 and 90,000 have been reported by others[16,19,20] and by the authors[17] by the measurement of the relative electrophoretic migration of the protein in polyacrylamide gels, in the presence of sodium dodecylsulfate, or by determination of its elution volume from gel filtration in guanidinum hydrochloride.

If it is assumed that the molecular weight of the subunit is 86,000, then the native enzyme is a tetramer of 344,000 mol wt.

Further support for the tetrameric structure of aspartokinase I-homoserine dehydrogenase I is provided by cross-linking the subunits with dimethylsuberimidate, followed by disc gel electrophoresis in dodecylsulfate.[17] When the enzyme was cross-linked at pH 8.5 in the presence of 0.3 *M* KCl, only 4 bands were seen, with the mobilities expected for the monomer, dimer, trimer, and tetramer of the subunits. At high reagent concentrations, a minor band, presumably the octamer, was observed.

Identity of Subunits

As the protein is known to carry two enzymatic activities, it is of interest to determine whether or not its subunits are identical. We have already seen that the subunits are equivalent in mass. Isoelectric focusing, in the presence of 6 *M* urea and 0.1% nonionic detergent Brij 35, gives a single peak with no indication of charge heterogeneity in the subunits.[17]

The amino acid composition (Table 2) of the carboxymethylated aspartokinase I-homoserine dehydrogenase I has been determined by us[17] and

TABLE 2

Amino Acid Composition of Aspartokinase I-Homoserine Dehydrogenase I

Amino acid	Residues/344,000 daltons
Lysine	126
Histidine	53
Arginine	178
Aspartic acid	311
Threonine	122
Serine	215
Glutamic acid	346
Proline	153
Glycine	245
Alanine	351
Half-cystine	42
Valine	254
Methionine	75
Isoleucine	172
Leucine	327
Tyrosine	69
Phenylalanine	118
Tryptophan	16

others,[16] and has been found to be in reasonable agreement with the one that we previously published for the nonalkylated protein.[1] In particular, the value of 10.8 ± 0.6 carboxymethylcysteines found per 86,000 mol wt agrees with the cysteic acid content obtained after performic acid oxidation (10.1 ± 0.3). It is important to note that no cysteine was found, showing that the carboxymethylation of the cysteine residues was complete. Electrophoresis at pH 2.1 of an acid hydrolyzate of the protein, carboxymethylated with radioactive iodoacetic acid, gave carboxymethylcysteine as the only radioactive spot, showing that carboxymethylation occured only on cysteine residues.

The tryptophan content of the protein, determined from its ultraviolet absorption in 6 *M* guanidinium chloride, is 3.9 ± 0.15 residues/86,000 mol wt. The number of tryptophan residues found by reaction with 2-nitrosulfenyl chloride agrees with this value.[17]

The results of chemical studies[17,21] strongly support the conclusion that the subunits are identical:

1. 50 to 52 tryptic peptides were detected by ninhydrin staining of the tryptic map of the soluble peptides. Fingerprint of the ^{14}C-labeled protein (obtained from bacteria grown on ^{14}C-labeled *Chlorella vulgaris* protein hydrolyzate) did not show additional peptides. As this soluble fraction contains 75 to 80% of the protein with the same lysine and arginine content as the total protein, 59 to 61 tryptic peptides were expected, assuming a molecular weight of 86,000. The slight difference between the expected and the obtained value is likely to result from superimposition on the map of distinct peptides.

2. The determination of the number of unique sequences around specific residues supplies the additional information that these sequences are present in all subunits.

Four unique sequences have been determined around tryptophan residues in distinct tryptic peptides from the soluble tryptic digest:

T85 Ala-Asp-Ile-CMCys-Glu-Trp-Thr-Asp-Val-Asp-Gly-Val-Tyr-Thr-CMCys-Asp-Pro-Arg

T10′ (Asx_3,Thr,Glx_5,Pro,Gly,Ala_2,Val,Leu_4,His,Trp)Lys-

TB1 Gln-Ser-Trp-Leu-Lys

TB2 Thr-Leu-Ser-Trp-Lys

No tryptophan containing peptide is found in the insoluble tryptic core.

Nine unique carboxymethylcysteine residues have been found, whereas the theoretical number for identical subunits was 10 or 11 (7 residues have been identified in the soluble tryptic digest (Figure 13a); two residues were identified in the insoluble tryptic core after chymotryptic and pronase digestion).

T60 Tyr-Val-Gly-Asx-Ile-Asx-Glx-Asp-Gly-Val-CMCys-Arg

T30 Leu(CMCys,Asx_3,Thr,Ser_2,Glx_2,Gly,Ala_2,Val_3,Ile,Tyr)

T10 (Asx_2,Ser_2,Glx,Pro,Gly_2,Ala_2,Val,Ile,Leu_3,His)Ile-CMCys-Arg

TNa Val-CMCys-Gly-Val-Ala-Asn-Ser-Lys

TNb Thr(Thr,Glu,Pro,Ala,Ile,Phe-Gln(CMCys,Pro,Ile_2,Leu)Lys

Ch60* CMCys-Val-Pro-Glx(CMCys,Asx_2,Ser,Glx,Val_2)Arg

*Chymotryptic peptide from the insoluble core.

It is difficult to rule out definitely either the existence of one or two additional carboxymethylcysteine peptides that may have escaped our investigation, or that a given peptide occurs more than once in the sequence. In any case, many more peptides would have been found, had the subunits been different.

3. Due to the courtesy of Drs. Hermodson, Walsh, and Neurath, the amino-terminal sequence of the protein was determined with an automatic sequenator up to 8 residues, and extended by Dr. John Walker, of our Institute, up to 17 residues.

At each turn of the sequenator, a single amino acid derivative was released, showing that all subunits share at least the same amino terminal sequence.

The C terminal sequence, Leu-Gly-Val-COOH, was determined by treatment with carboxypeptidase A, with roughly 1 equivalent of each residue being released per 86,000 g.[17]

Crystal Structure

The aspartokinase I-homoserine dehydrogenase I has been crystallized from ammonium sulfate in the presence of threonine and NADPH.[21a] The tetragonal crystals (Figure 5a) have been found by X-ray diffraction to belong to space group I 422 or I $4_1$22 with an asymmetric unit of 1.0×10^6 Å^3. Assuming that the solvent occupies 53% of the total volume, this asymmetric unit will contain 1 tetrameric molecule.[21a]

The symmetry of this crystal form does not, therefore, express the possible symmetrical arrangement of the four subunits.

Stoichiometry of Ligand Binding

The bifunctional protein has numerous ligands. In addition to its substrates, L-aspartate, ATP, homoserine, aspartate semialdehyde, and reduced and oxidized triphosphopyridine nucleotide, the allosteric effector L-threonine must bind to the protein.

The feasibility of determining the stoichiometry of binding depends, in the last analysis, on the association constants of the ligands. In some instances, it has been necessary to establish the stoichiometry by covalent affinity labeling or by changing the usual conditions in order to increase the association constants. Whether or not the values found correspond to binding at the specific sites will be discussed in these cases.

NADP and NADPH Binding

Three methods have been used: equilibrium dialysis, fluorescence studies, and circular dichroism.

The binding of the oxidized coenzyme was studied by gel filtration of the protein, using

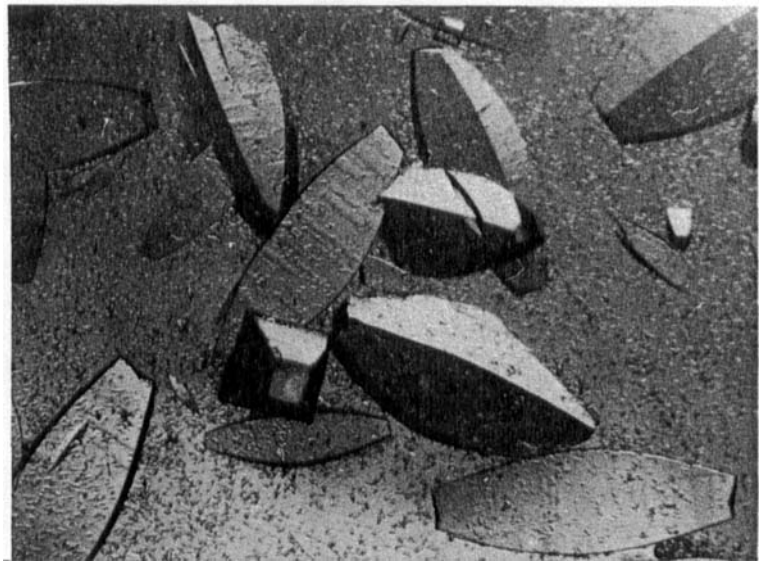


FIGURE 5a. Tetragonal crystals of aspartokinase I-homoserine dehydrogenase I. The larger crystals are about 1 mm long.

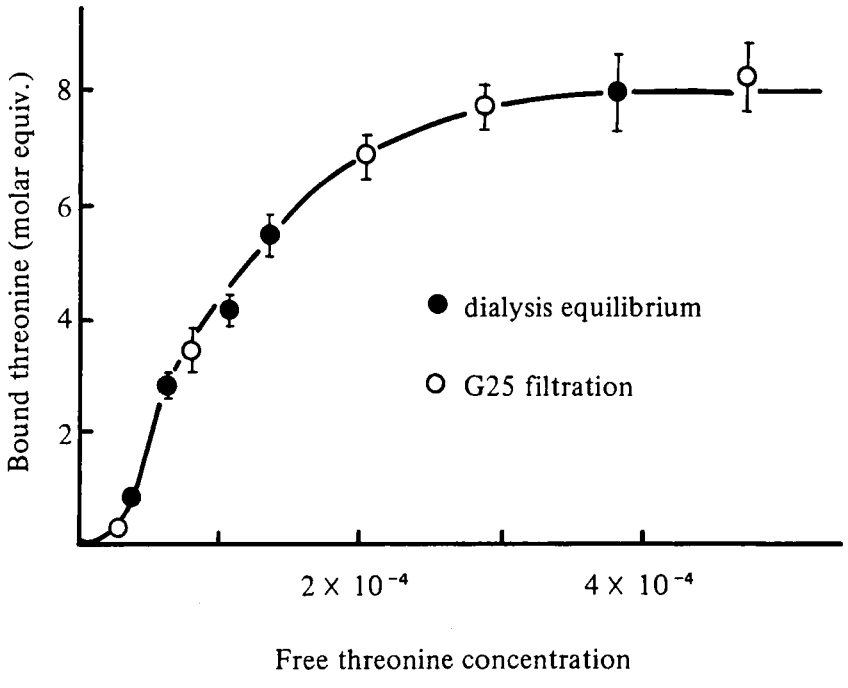


FIGURE 6. Binding of L-threonine to aspartokinase I-homoserine dehydrogenase I.

(carboxyl-^{14}C) nicotinamide dinucleotide phosphate, on Sephadex G-50 equilibrated with the radioactive ligand. The linear Scatchard plot indicates that the ligand binds according to a single adsorption isotherm.[17,22]

Extrapolation leads to a value of 4 ± 0.25 mol of $NADP^+$ bound per mol of enzyme,[17] and the affinity constant is close to 12 μM in the buffer used. It should be noted that the variation of homoserine dehydrogenase activity is also Michaelian, the apparent dissociation constant being ten times higher (K_m $NADP^+$ = 110 μM).[9]

The absorption at 340 nm was used to detect the reduced coenzyme upon elution from Sephadex columns equilibrated with the ligand. Satisfactory values of the number of bound molecules were obtained near 23°C, at saturating concentration of NADPH and in the same buffer as above. These values are between 2.7 and 4.1 molar equivalents, with an uncertainty of about 15%. They do not vary significantly when 1 mM L-threonine is added, or when KCl concentration is varied from 0.15 to 0.65 M; moreover, addition of the substrate of the reverse reaction, L-homoserine, does not lead to higher values.

NADPH binding to enzyme results in the appearance of an extrinsic dichroic band at 348 nm. The change in molecular ellipticity was used to titrate the protein with NADPH to a 15-fold higher ligand concentration than was possible with fluorometric methods. The number of sites found, 4 ± 0.4/tetramer,[2,17] was in agreement with the fluorometric determination, based on the considerable increase of the blue fluorescence of the coenzyme upon addition of aspartokinase I-homoserine dehydrogenase I.[17,22]

Threonine Binding

The experiments using L-(^{14}C)-threonine were performed by equilibrium dialysis and gel filtration.[2,17,22] Figure 6 shows that at 23°C and in a buffer containing 0.15 M KCl, saturation of the binding sites was reached at free ligand concentrations in the 0.3 to 0.5 mM range, where 8.1 ± 0.4 mol of threonine are bound to 1 mol of enzyme. Under these conditions, only 10 to 15% of the ligand is bound to the protein, but the value at saturation was found to be reproducible within 5%. Moreover, essentially the same values were obtained with aged samples of protein which had lost 30% of their homoserine dehydrogenase activity; these preparations lost none of their binding capacity towards the allosteric effector, and the remaining activity was normally inhibited by threonine. On the other hand, treatment by N-ethylmaleimide, as well as by DTNB or pMB,

TABLE 3

Binding of Threonine

Additions	Temperature (°C)	Source of data	Concentration of free L-threonine (m*M*)	Molar equivalents of bound threonine
0.15 *M* KCl (buffer B)	4	Dialysis	0.16	6.9
			0.86	7.6
	23	Filtration	0.20	6.8
	27	Dialysis	0.13	5.5
			0.38	8
0.5 *M* KCl	23	Filtration	0.20	3.8
			0.30	7
			0.50	6.9
0.15 *M* KCl + 25 μ*M* NADPH	20	Filtration	0.37	7.5
0.15 *M* KCl + *N*-ethylmaleimide treated protein	22	Filtration	0.30	0

Data were obtained from equilibrium dialysis or from Sephadex gel filtration.

desensitizes the homoserine dehydrogenase activity; the modified protein no longer binds threonine.

Since the inhibition by threonine of the homoserine dehydrogenase[12] and the aspartokinase[11] activities is strongly cooperative, it is interesting to find that the binding of threonine also shows cooperativity.

Table 3 shows that at saturation the number of molar equivalents of bound threonine is essentially 8 at different temperatures and in the presence of 0.15 *M* or 0.5 *M* KCl. However, at nonsaturating concentrations of free threonine, the extent of binding depends on a number of factors, especially the concentration of KCl.[22] High concentrations of KCl, which diminish the binding of threonine, have been found to displace in a very similar manner the inhibition curves of the homoserine dehydrogenase[4] and aspartokinase[5] activities. In the presence of aspartate, the binding of threonine is also found to decrease,[22] a result which could be expected, since the inhibition of the aspartokinase is competitive towards aspartate.[8]

Binding of ATP and 6-Mercapto-9-β-ribofuranosyl-5'-triphosphate (SHTP)

The stoichiometry of ATP binding to the enzyme was not readily established because of the low affinity of this ligand to the protein.

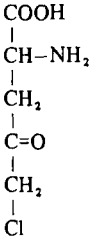
Advantage has been taken of the existence of SHTP, an adenosine triphosphate analog, which is a substrate of the aspartokinase activity.[24] Incubation of the enzyme with SHTP in the absence of L-threonine leads to a modified enzyme of essentially the same molecular weight as the native protein which has specifically lost its aspartokinase activity; however, the modified enzyme is desensitized to threonine. Reaction of the modified enzyme with mercaptoethanol releases four molecules of SHTP per molecule of the protein.[17,24] Saturating levels of threonine protect the enzyme against SHTP. These results raise several questions, especially concerning the mechanism of covalent labeling and its specificity. The release of SHTP by mercaptoethanol indicates that a disulfide bond has been formed between the reagent and a sulfhydryl group of the protein. This suggests that the disulfide derivative of SHTP could be the reactive form. However, when the previously prepared disulfide (TPSSTP) is allowed to react with the protein, it modifies a great number of sulfhydryl groups, acting as an unspecific reagent analogous to *N*-ethylmaleimide or dithiobis-nitrobenzoic acid (Saari and Truffa-Bachi, unpublished results). We propose that the nucleotide triphosphate moiety of SHTP directs the analog near the catalytic site (it is a substrate), and is then oxidized *in situ* by the molecular oxygen dissolved in the medium and then reacts with a specific sulfhydryl group at or near the catalytic site.

ATP is not a good protector against SHTP inactivation, suggesting that either SHTP has a much greater affinity than ATP for the catalytic site or that it does not bind at this catalytic site, but that when covalently bound, it somehow interferes with catalysis. In any case, it is interesting to note that one equivalent of the analog is bound per subunit.

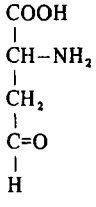
The binding of ATP to aspartokinase is fairly weak, as mentioned above; consequently, the errors involved in the direct determination of the binding constant and of the stoichiometry by equilibrium dialysis are fairly large. Erhlich and Takahashi[10] have attempted these measures, using ATP-γ-^{32} P in the absence of metal. The best computer fit of the data gives 4.6 ± 1.6 sites/tetramer and a dissociation constant of 2.8 ± 1.0 m*M*. However, this is an extrapolation, the maximum number of molecules of ATP experimentally found being 2.5/tetramer.

Affinity Labeling by 2-Amino-4-oxo-5-chloropentanoic Acid, an Analog of Aspartate Semialdehyde

The affinity of the protein for homoserine (a substrate for the homoserine dehydrogenase activity) is so low that the stoichiometry of its binding cannot be determined by equilibrium dialysis. Therefore, an affinity label has been sought which could allow such a determination. 2-Amino-4-oxo-5-chloropentanoic acid[25] is such a substance. Its structural formula is given below, compared to that of aspartate semialdehyde.



2-Amino-4-oxo-5-chloropentanoic acid



Aspartate semialdehyde

Incubation of the enzyme with this chloroketone in the absence of L-threonine leads to a loss of the aspartokinase activity and desensitizes the dehydrogenase to inhibition by threonine. In this respect, the reaction with chloroketone is similar to that obtained with many sulfhydryl reagents we have tested. However, when L-threonine is present, it is a very specific reagent, which inactivates the dehydrogenase and leaves the aspartokinase activity intact and sensitive to threonine.[26]

Evidence that 2-amino-4-oxo-5-chloropentanoic acid acts as an affinity label has been amply documented.[26] Suffice it to say that the inactivation of the dehydrogenase requires the covalent binding of one equivalent of the analog per subunit.

Table 4 summarizes the stoichiometries thus far established for various ligands of the bifunctional enzyme.

Availability of Sulfhydryl Groups

As has already been stated, sulfhydryl titration results in the loss of aspartokinase activity and the desensitization of the homoserine dehydrogenase activity to threonine inhibition. Modification of a single sulfhydryl per subunit by SHTP is sufficient to obtain this result, in which case the enzyme remains tetrameric.[24] Modification by less specific reagents, such as *p*-mercuribenzoate, *N*-ethylmaleimide, dithiobis (nitrobenzoic acid), or iodoacetamidosalicylic acid, leads to the same result; however, with these reagents many more sulfhydryl groups are titrated and the modified enzyme becomes dimeric, suggesting that sulfhydryl groups are somehow involved in the intersubunit association areas.

Figure 7 is an example of the course of titration by DTNB and of the concomitant events. Out of the 11 -SH groups present per subunit, 7 are titratable by DTNB, with the titration being accompanied by the destruction of the kinase and the desensitization of the dehydrogenase. It is noteworthy that during the titration with DTNB and all other sulfhydryl reagents tested, the remaining aspartokinase molecules are fully sensitive to threonine. In the presence of 2 m*M* threonine, essentially no sulfhydryls can be titrated.[1]

The above experiment was performed in the presence of 0.15 *M* KCl. If the concentration of K^+ ions is reduced to 5 m*M*, very different results are obtained: although the final number of titratable sulfhydryls is the same, titration is much more rapid, and threonine protects only 4 sulfhydryl groups; moreover, titration of the 3 -SH in the presence of threonine does not result in the loss of kinase or desensitization of the dehydrogenase.

TABLE 4

Stoichiometry of Binding

Ligands substrates	Method	Equivalents bound per subunit
ATP	Covalent binding of analog	1
	Equilibrium dialysis	1
L-Aspartate semialdehyde	Covalent binding of analog	1
$NADP^+$	Gel filtration	1
	Equilibrium dialysis (unpublished)	1
NADPH	Gel filtration	1
	Fluorimetry	1
	Circular dichroism	1
L-Threonine	Equilibrium dialysis	2
	Gel filtration	2

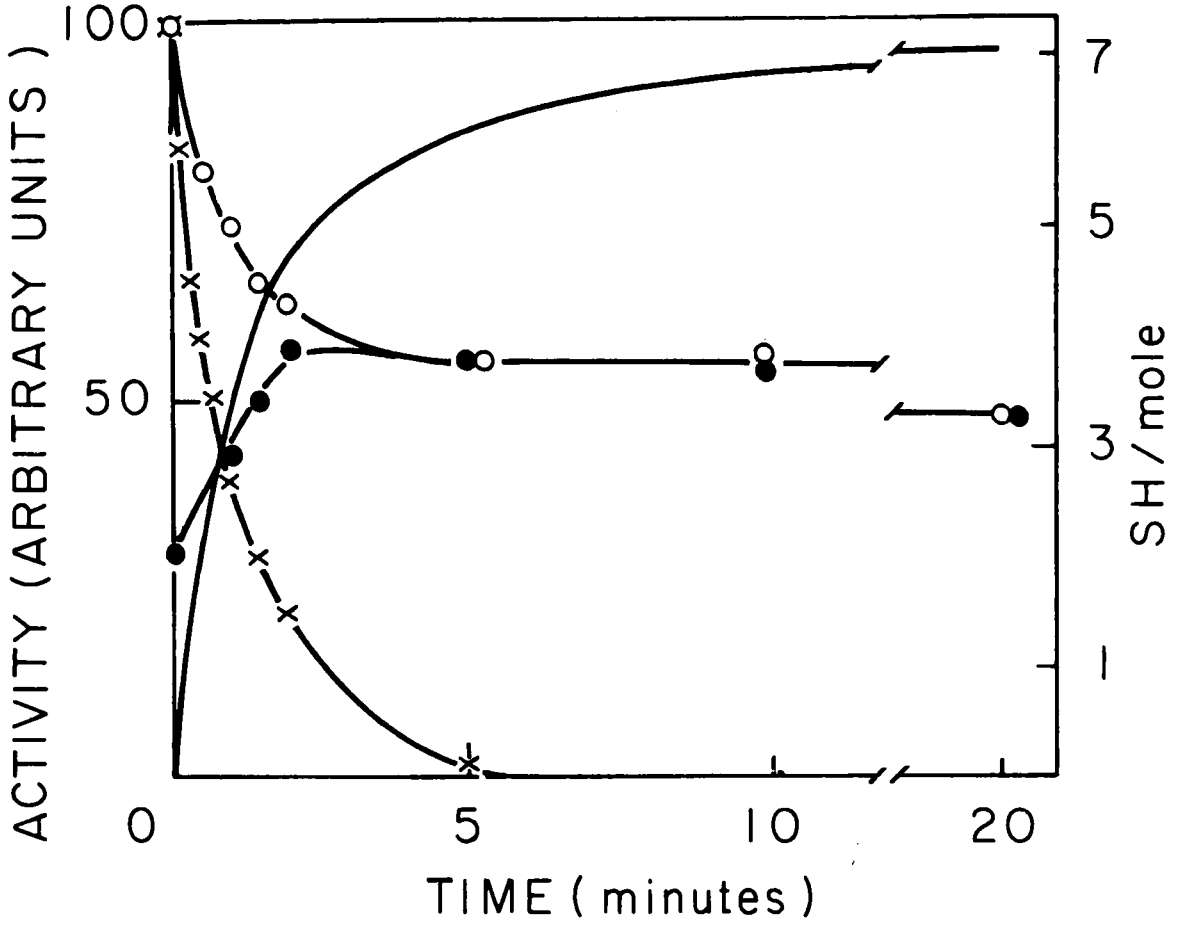


FIGURE 7. Titration of available -SH groups of aspartokinase I-homoserine dehydrogenase I by DTNB: concomitant inactivation of aspartokinase (X) and desensitization of homoserine dehydrogenase. The activity of the latter is measured without (○) and with (●) threonine. The solid line without symbols refers to the number of sulfhydryl groups titrated per mole of enzyme.

ATP plus homoserine affords the same protection as threonine.[7]

CONFORMATIONAL CHANGES

Conformational changes of proteins can be visualized by many different techniques. Two major theories have been proposed for the interpretation of conformational changes of oligomers: (1) the concerted allosteric theory proposed by Monod et al.,[13] which is based on the displacement, observed upon addition of a ligand, of a preexistent equilibrium, and (2) the sequential model of Koshland et al.[27] which postulates that the change is induced upon ligand binding.

The curves of the inhibition by threonine of aspartokinase and homoserine dehydrogenase activities show that cooperative homotropic effects are produced by addition of this ligand (Figure 3). Physicochemical and immunological techniques have been used for the study of the conformational changes of the aspartokinase I-homoserine dehydrogenase I of *E. coli* K12.

Difference Spectra

The spectrophotometric comparison of 2 identical solutions of protein, one in a phosphate buffer containing 0.15 *M* KCl and the other in the same buffer containing 0.15 *M* KCl plus 0.5 m*M* L-threonine, shows a difference spectrum in the 250 to 310 nm range (Figure 8). The complexity of the spectra indicates that several aromatic residues are involved. From these spectra, two conformations of the enzyme have been postulated: a T form, characterized by the fact that the aromatic residues are buried, and an R form, with exposed aromatic residues.[2,22]

The same, spectrophotometrically defined, T form can be obtained either in the absence of KCl[2,22] or by addition of serine in the presence of KCl.[15] The equilibrium can be shifted back to the R form by addition of aspartic acid.

Protein Fluorescence

The change in the absorption spectrum of the protein upon addition of threonine in the presence of KCl 0.15 *M* is associated with a quenching of the fluorescence of the protein.[22]

From the studies of the excitation and emission spectra of the protein, it was concluded that, when the excitation was performed at wavelengths below 280 nm, the results were in agreement with the two-state model; however, when the excitation was performed at 295 nm (in the absorption band of tryptophan), the effects of ligands could not be accounted for by the two-state model.[2] The binding of NADPH to the protein can be monitored by the fluorescence of transfer upon excitation of the protein in the 295 nm region and observation of the fluorescence at 450 nm. Addition of threonine results in a quenching of the fluorescence of transfer.[22]

Recently, we have studied the effect of serine on the fluorescence of the protein, and results analogous to the ones obtained with threonine were found; in contrast, serine causes an increase of fluorescence transfer from the protein to NADPH.[15] Figure 9 summarizes some of the data obtained in our fluorescence studies.

Dichroic Circular and ORD Spectra

Circular dichroic studies have shown that the spectrum of the protein is characterized by a complex overlap of negative bands. These bands arise from aromatic transition and possibly, at shorter wavelengths, from disulfide bonds. The binding of threonine to the enzyme results in a decrease of the magnitude of the ellipticity of the bands (Figure 10). The change in ellipticity at 280 nm was used to study the conformational change of the protein upon addition of threonine. The curve is sigmoidal and can be superimposed on that obtained using other physicochemical methods.[23] The circular dichroism change at 280 nm is essentially measuring the state function. The binding of aspartic acid in the presence of 0.15 *M* KCl results in a small decrease at the long wavelength side of the near ultraviolet dichroic spectrum of the protein; this can be interpreted either as a specific interaction of aspartate with the protein or as a local conformational change resulting from aspartate binding. NADPH binding can be followed by the appearance of an extrinsic dichroic band at 348 nm. Addition of threonine results in the diminution of the ellipticity of the extrinsic dichroic band.[23]

Hydrogen Exchange

Measurement of the hydrogen exchangeable with tritiated water in the presence or absence of the allosteric effector results, at threonine saturation, in a 40% increase in the number of tightly sequestered hydrogens. The results show an increase in core size upon the binding of threonine,

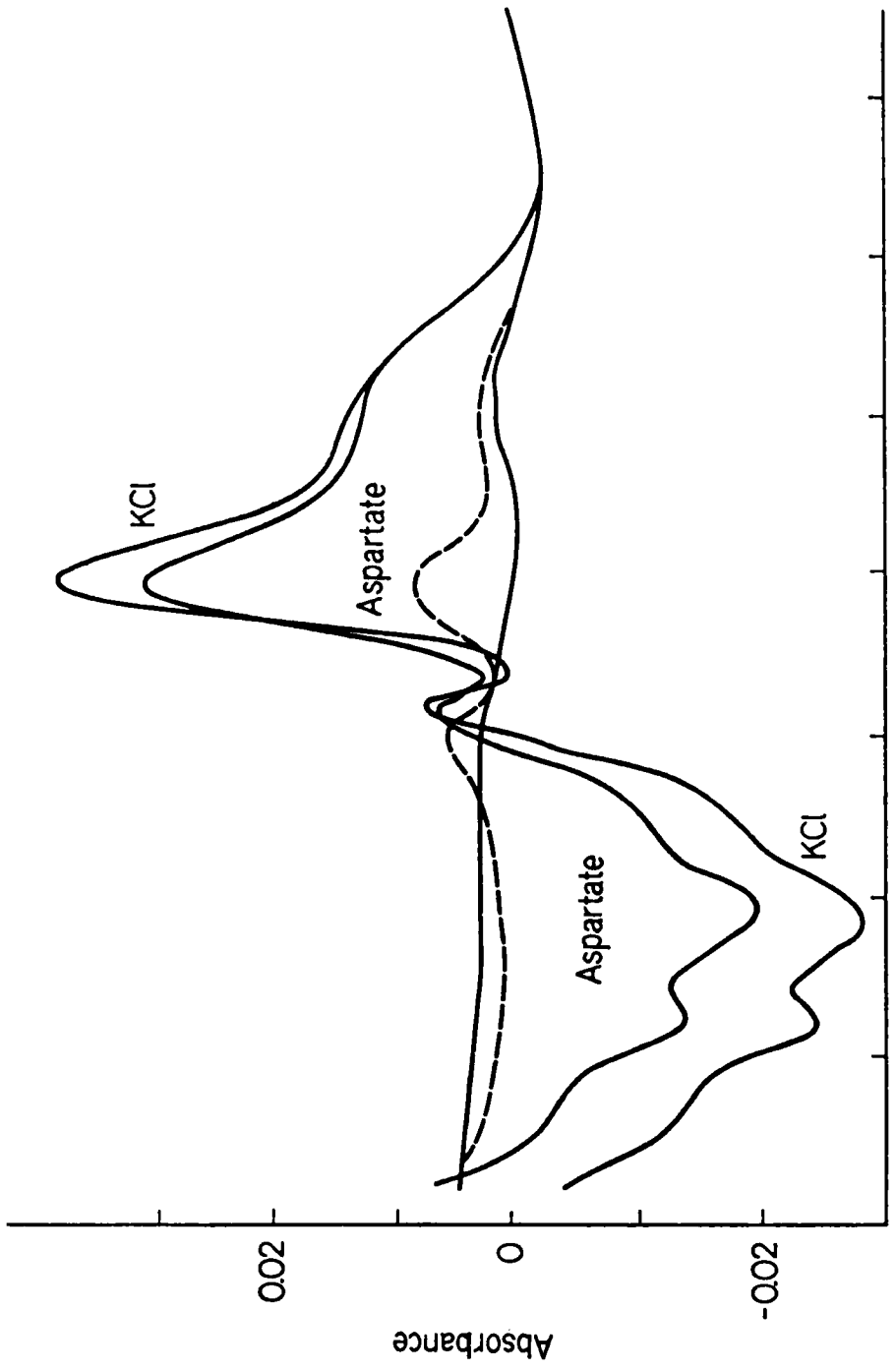


FIGURE 8. Difference spectra in the presence of threonine. Baseline: reference and sample, no addition. Dashed line: reference, no addition; sample, 0.5 m*M* L-threonine. "Aspartate" spectrum: reference, 15 m*M* L-aspartate; sample, 15 m*M* L-aspartate and 0.5 m*M* L-threonine. "K+" spectrum: reference, 0.5 *M* KCl; sample, 0.15 *M* KCl and 0.5 m*M* L-threonine.

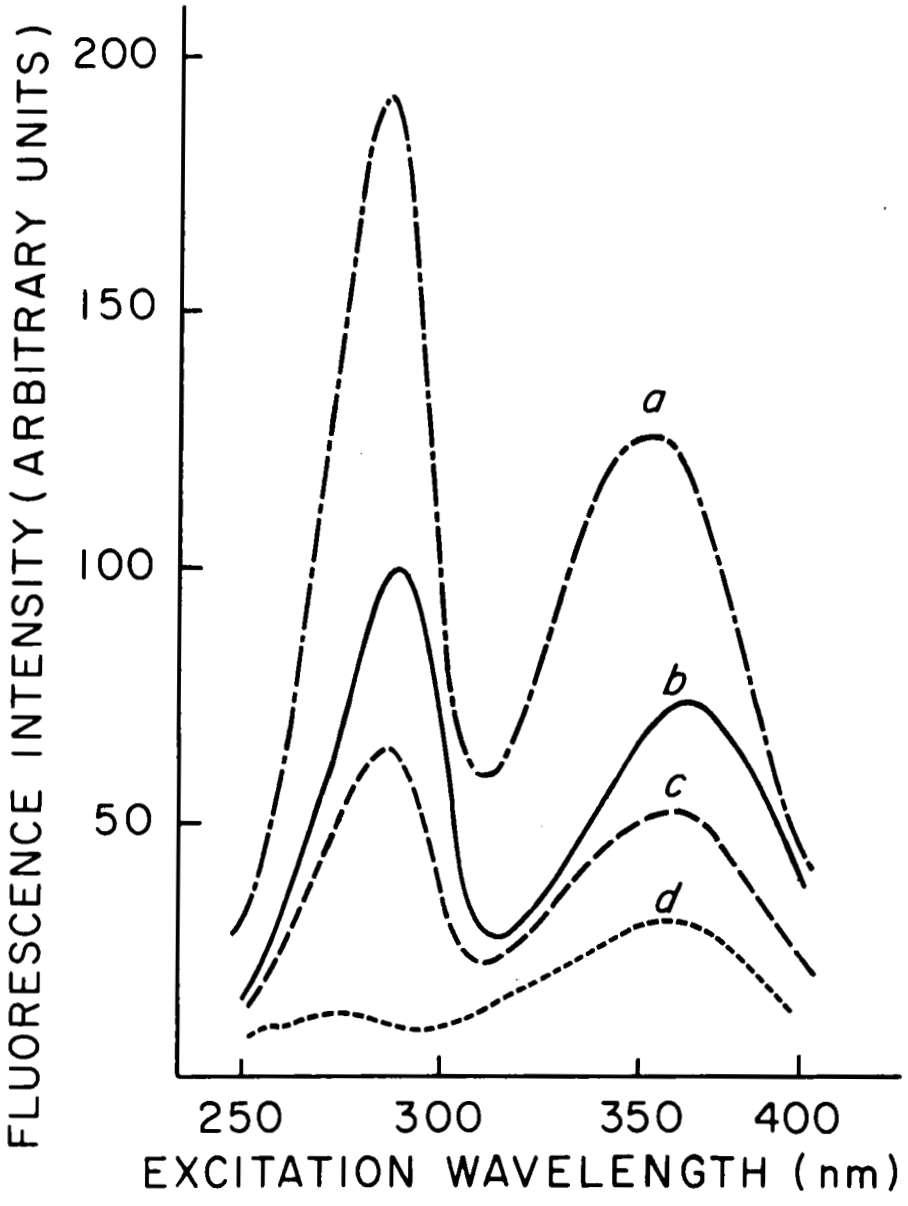


FIGURE 9. Fluorescence of the protein-NADPH complex. The emission is recorded at 450 nm. NADPH: 3 μM. Protein: 1.5 μM. (a) 10 mM L-serine; (b) No ligand; (c) 1 mM L-threonine: (d) Fluorescence of 3 μM NADPH in the absence of protein.

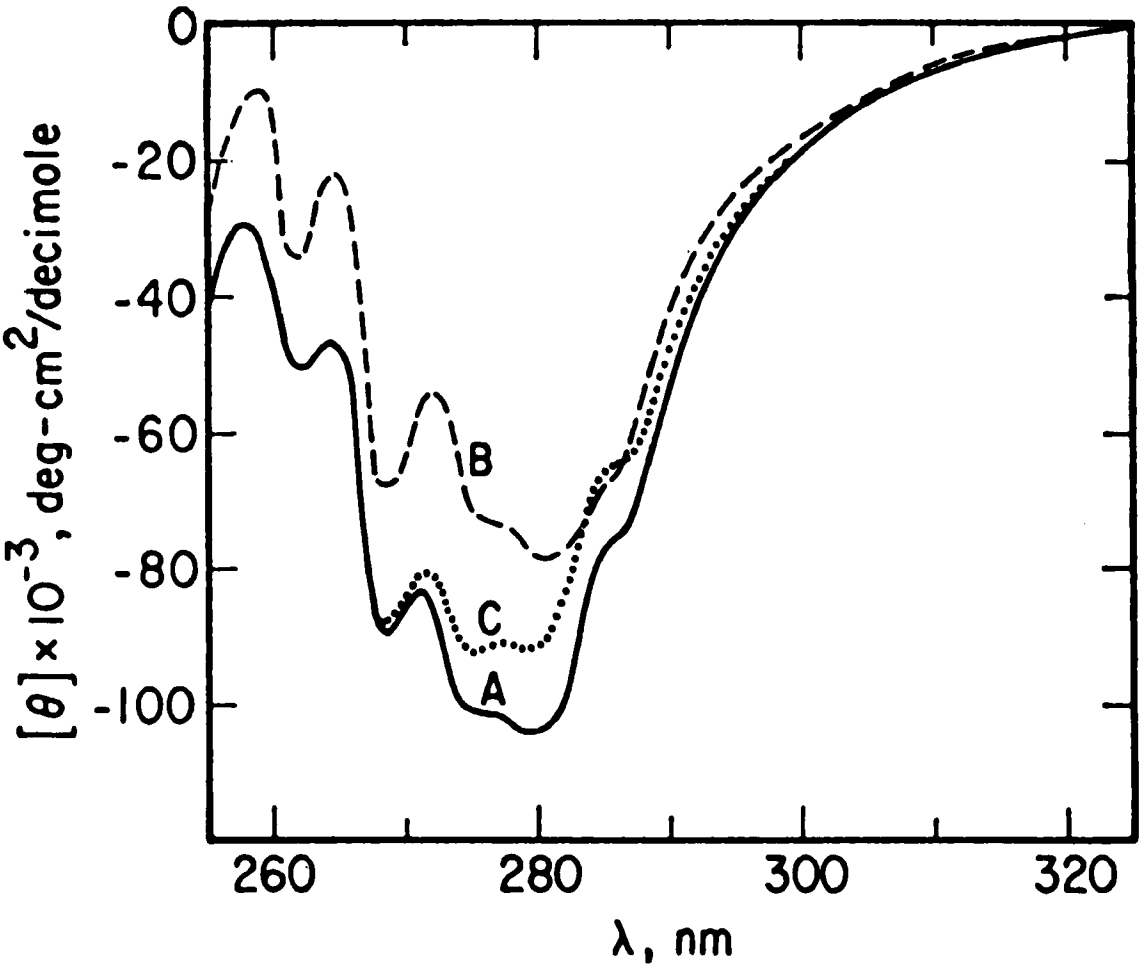


FIGURE 10. Near ultraviolet circular dichroic spectra of aspartokinase I-homoserine dehydrogenase I. A: Free enzyme. B: Enzyme + 2 m*M* L-threonine. C: Enzyme + 20 m*M* L-aspartate.

indicating a tighter structure with the hydrophobic interior of the enzyme becoming less accessible to the solvent, which agrees with the ultraviolet difference spectral changes.[28] However, no definitive statement can be made on the absolute size of the perturbation of the molecule that occurs.

Reaction with Specific Antibodies

Two conformations of the enzyme are distinguished by the antibodies against aspartokinase I-homoserine dehydrogenase I: in the presence of aspartate and K^+, the fixation on a immunoadsorbent column made from antiaspartokinase I-homoserine dehydrogenase I immunoglobulins is about 50% less than in K^+ alone or in K^+ plus threonine.[29]

The form which predominates in the presence of threonine, called T form in our previous publications,[2] must have some antigenic determinants exposed to react with the corresponding sites on the antibodies, whereas they must be less available in the presence of aspartate – that is, in the R form. However, the transition from the T to the R form can be achieved not only with aspartate, but also with K^+ ions; the concentration of K^+ in buffer A is such that the protein is already in the R form (as defined by its spectroscopic properties) before aspartate is added. Therefore, we can define the following equilibria:

$$R_2 \underset{\text{aspartate}}{\rightleftharpoons} R_1 \overset{\text{threonine}}{\underset{\text{KCl}}{\rightleftharpoons}} T \qquad (3)$$

In this model, R_1 and R_2 have identical or

similar spectroscopic or hydrogen exchange properties, since KCl and aspartate bring about the same variation of these properties. On the other hand, the conclusion that R_1 and R_2 differ by their reactivity to their specific antibodies is inescapable.

At the pH where the adsorptions are performed, the enzyme remains tetrameric, whatever the ligand may be.[30] Thus, the above equilibria are not between different states of polymerization of subunits, but between authentic conformational isomers.

Discussion

We have seen that various techniques can be used to visualize the conformational changes that the aspartokinase I-homoserine dehydrogenase I undergoes upon the binding of different ligands. This binding displaces a preexisting equilibrium.

By spectroscopic studies it has been shown that, in the absence of ligands, the majority of the molecules of the enzyme (more than 90%) are in the T form.[2] This form is stabilized by addition of threonine[2,22] or serine.[15] The other form, the R form, is stabilized by aspartate or K^+ ions.[2,22]

Taking into account the fact that the addition of threonine to the enzyme in the T form does not change the spectroscopic properties of the enzyme and that the addition of aspartate or K^+ has the same effect on the optical properties of the enzyme, Janin and Cohen[2] concluded that the spectrometric methods probe only the conformations and not the binding of the ligands themselves.

Pure T and R (as defined by their optical properties) forms can be obtained by the addition of the appropriate ligand, and the measurement of the change of the ratio of R to T form with ligand concentration shows that the enzyme responds in a concerted manner to ligand binding.

From our work[2,17,22] and studies by Heck,[31] it can be assumed that the allosteric transition is totally concerted within the tetramer.

However, all the properties of the aspartokinase I-homoserine dehydrogenase I cannot be explained by this simple two-state model, and a series of data shows that the number of species is, indeed, higher.

Stopped flow and temperature jump experiments[32] show that the T and the R forms and at least one more species of the protein exist in the absence of ligand. T-jump experiments show that 2 processes with different time constants (10^3 sec^{-1} and 15 sec^{-1}) take place, with the slower constant corresponding to the relaxation of the T-R equilibrium.

Upon addition of ligands, K^+, aspartate, serine or threonine, or ATP plus homoserine, one observes different reactivities of sulfhydryl groups or accessibility to antibodies, which show that the liganded T or R forms may behave differently from the unliganded species, and even that a liganded species might behave differently according to the probe tested.

THE SUBUNIT OF ASPARTOKINASE I-HOMOSERINE DEHYDROGENASE I IS COMPOSED OF TWO STRUCTURAL REGIONS, EACH CARRYING ONE OF THE TWO CATALYTIC ACTIVITIES

The HDH Fragment

Effect of Limited Proteolysis of Aspartokinase I-Homoserine Dehydrogenase I

As shown in Figure 11 (upper part), limited proteolysis by α-chymotrypsin (1% w/w) leads to desensitization of the homoserine dehydrogenase activity, whereas the kinase activity is completely destroyed. At any given time before complete inactivation, the remaining kinase is fully sensitive to threonine.[33]

The analysis of the products of proteolysis on SDS gels (lower part, Figure 11) shows the disappearance of the band corresponding to the native enzyme (mol wt = 86,000) correlated to the appearance of a faster band, of mol wt = 55,000; when followed on nondenaturing gels (Figure 12), a third band (D′) is transiently present, corresponding to an intermediate in the proteolysis of the native enzyme E to the final product F.[33,34]

Purification and Molecular Weight of the Proteolytic Fragment

In order to characterize the products of the proteolysis, the mixture corresponding to the pattern of gel 5 in Figure 11 was purified by gel

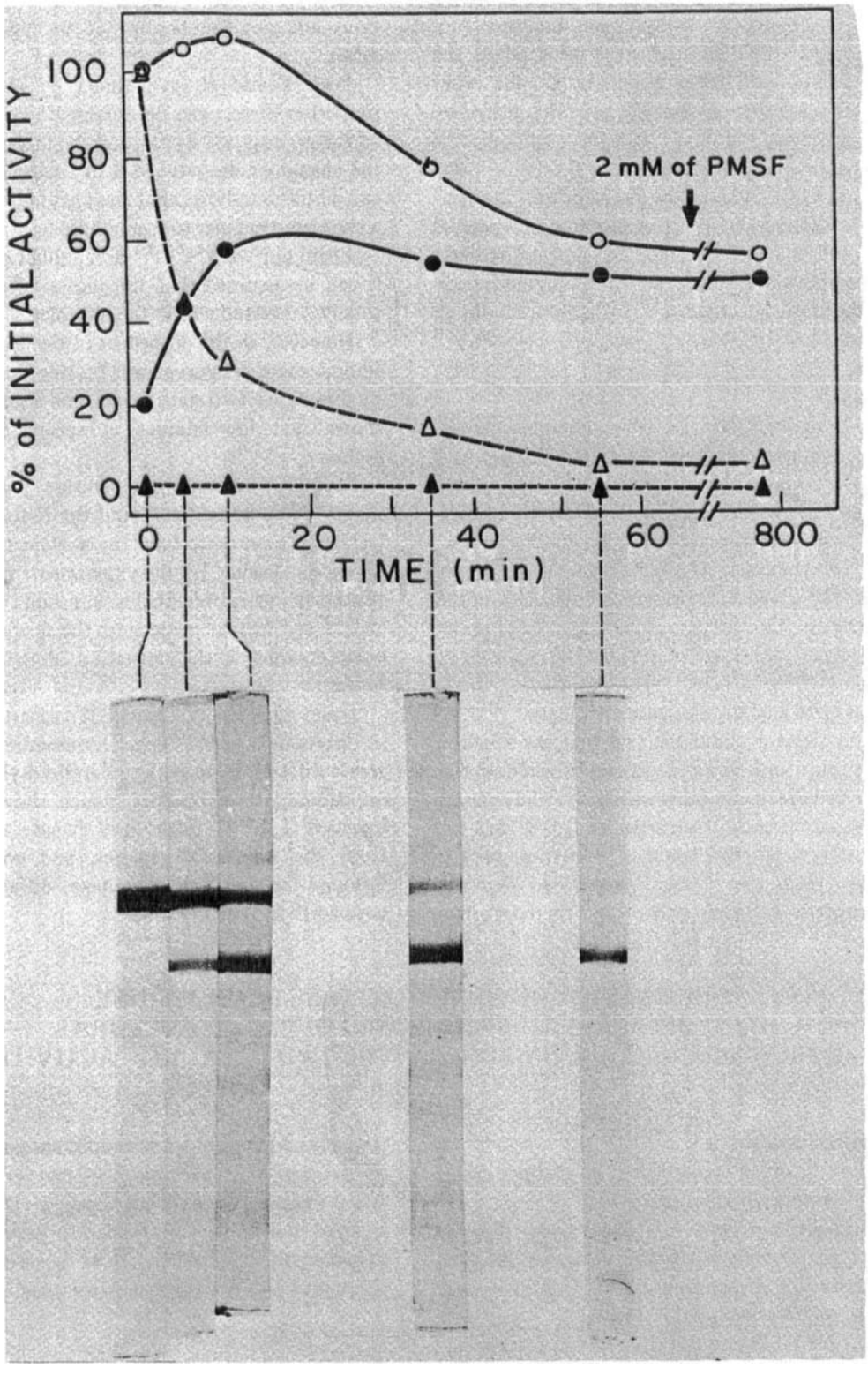


FIGURE 11. Effect of proteolysis on aspartokinase I-homoserine dehydrogenase I. Upper part: enzymatic activities: Δ: aspartokinase activity; ○: homoserine dehydrogenase activity: ●: homoserine dehydrogenase activity measured in the presence of 2 m*M* L-threonine. Lower Part: electrophoresis on dodecylsulfate gels.

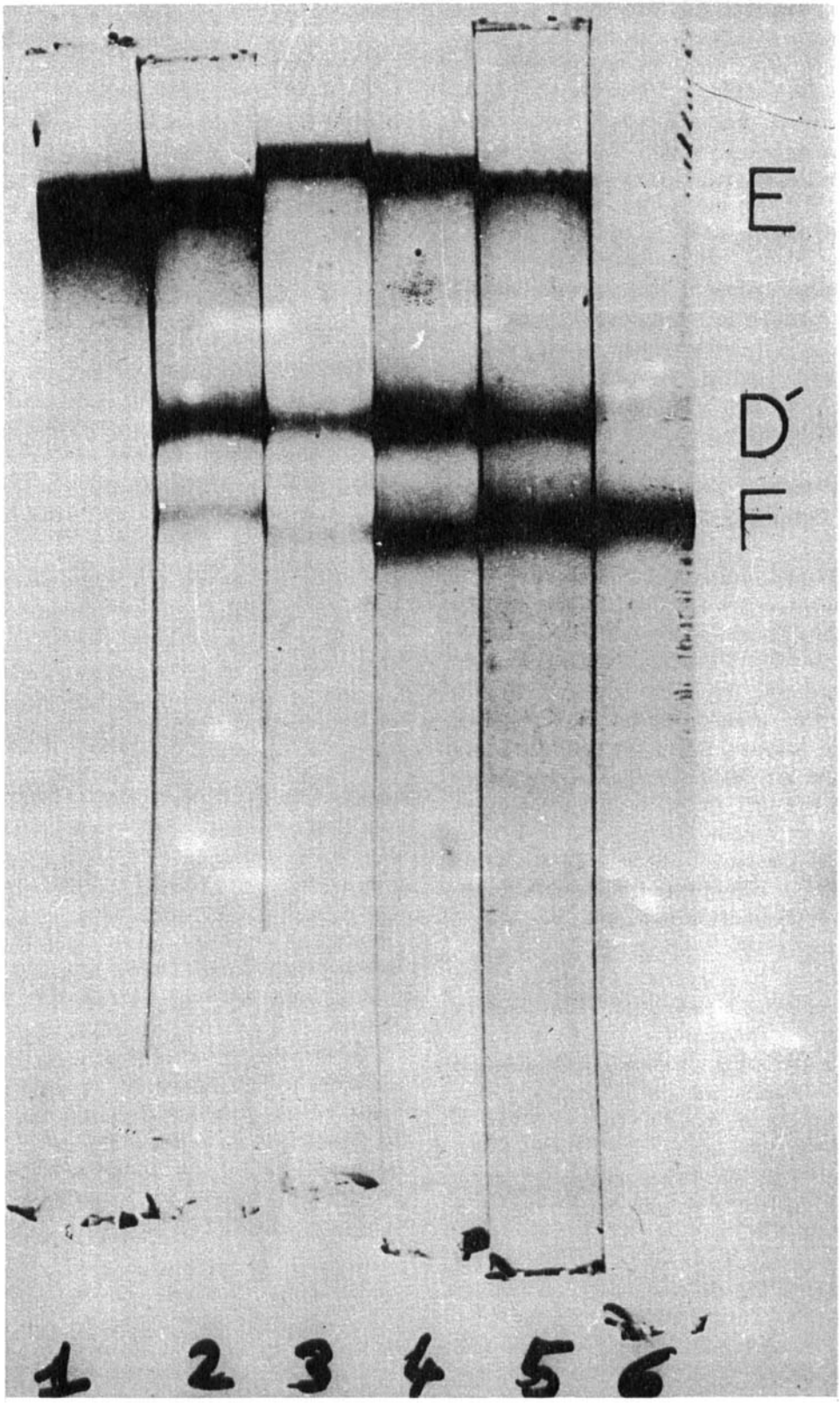


FIGURE 12. Effect of proteolysis on aspartokinase I-homoserine dehydrogenase I. The nondenaturing gels are stained for homoserine dehydrogenase activity.

filtration. F was separated from D′ and undigested enzyme; the comparison of the molecular weight of D′, determined from its elution from the column and from SDS gels, shows that it is a dimer having a subunit molecular weight very close to that of the native enzyme (86,000) and possessing both activities partially sensitive to threonine inhibition.[33,34] F, the purified final product of proteolysis, has a molecular weight of 110,000, as determined by its elution volume and by equilibrium sedimentation.[33] We call this dimeric fragment (subunit mol wt = 55,000), which has retained only a desensitized homoserine dehydrogenase activity, the HDH fragment.

It has a sedimentation coefficient of 6 S, determined by sucrose gradient centrifugation[33] and analytical ultracentrifugation.[35] This value is consistent with the rough, globular shape of the fragment, assuming a molecular weight of 110,000.

When trypsin, papain, or subtilisin was used as the proteolytic agent instead of α-chymotrypsin, the same effects were observed; the loss of kinase activity, the dehydrogenase desensitization, and the characteristic band pattern upon electrophoresis were obtained in all cases. With all proteolytic enzymes tested, the HDH fragment proved to be very resistant to further degradation. This indicates that the nature of the fragment obtained by proteolytic cleavage is imposed by the structure of the protein substrate (aspartokinase I-homoserine dehydrogenase I) rather than by the specificity of the protease itself.

Localization of the Subunit of the HDH Fragment on the Complete Polypeptide

The characterization of the C terminal residues of the HDH fragment was made by treatment with carboxypeptidase A. Roughly one equivalent of valine, leucine, and glycine per 55,000 g was released. With the same conditions the C terminal sequence of the native enzyme was shown to be Leu-Gly-Val-COOH.[33]

The N terminal residue of the HDH fragment was determined by dansylation.[33] This residue varies, depending on the proteolytic enzyme used, but in no case was a methionine observed (the amino terminal residue of the native enzyme is a methionine).

These experiments prove that the polypeptide chain of the HDH fragment corresponds to the C terminal part of the complete polypeptide chain of aspartokinase I-homoserine dehydrogenase I.

Figure 13b shows the autoradiograph of the fingerprint of the soluble tryptic peptides of the HDH fragment, after carboxymethylation of the cysteine residues with ^{14}C iodoacetic acid: the fragment contains only three of the six soluble peptides present in the native enzyme: T60, T30, and TNa.

Kinetic Parameters

The K_m of the HDH fragment for its substrates NADPH and aspartate-β-semialdehyde are of the same order as those for the native enzyme: K_m NADPH = $6.10^{-5}M$; K_m ASA = 0.2 m*M*. The specific activity expressed per mol of subunit is about one half of that of the native enzyme, based on a molecular extinction coefficient of 37,000 for a molecular weight of 55,000.[36]

Threonine Binding

Threonine does not inhibit the activity of the HDH fragment at 2 m*M*, where the maximum inhibition is obtained with the native enzyme.

In contrast with the situation observed with native enzyme, the transfer fluorescence of bound coenzyme on the HDH fragment is not quenched by 10 m*M* threonine; at this concentration, threonine has no effect on the number of sulfhydryl groups titrated by DTNB. Finally, equilibrium dialysis with ^{14}C-threonine shows that the HDH fragment does not carry any threonine binding site with an affinity constant better than $10^{-3}M^{-1}$ (the dialysis was carried out at 20 m*M* K^{+4}). All these experiments prove that no fixation of threonine occurs with the HDH fragment under conditions analogous to those where this ligand binds to the native protein. The HDH fragment thus seems to be "frozen" in an active conformation which is unable to afford the conformational change necessary to achieve the inhibition of the activity.[36]

However, an inhibition of this activity is observed at high threonine concentration (K_I = 40 m*M*). The competitive nature of this inhibition indicates that it is likely to correspond to a binding of threonine to the homoserine dehydrogenase catalytic site. This interpretation is consistent with the inhibition of the activity of the fragment by L-serine.

TABLE 5

Kinetic Parameters of the Aspartokinase Fragment

K_m L-aspartate	= 6 m*M*
K_m ATP	= 3 m*M*
Turnover number of the tetrameric enzyme	= 1,700 mol aspartyl-phosphate synthesized × min^{-1} × mol $enzyme^{-1}$
Turnover number of the monomeric enzyme	= 1,425 mol aspartyl-phosphate synthesized × min^{-1} × mol $enzyme^{-1}$

Stability of the HDH Fragment

In contrast to the behavior of the native enzyme, the state of aggregation of the HDH fragment is not affected by KCl or threonine; it remains a dimer even in the absence of these ligands, whereas the native tetramer tends to dissociate into dimers under these conditions.[30] Moreover, it has been shown that at pH conditions where the native enzyme dissociates into dimers, the fragment does not dissociate into monomers.[35]

Conclusion

The existence of the HDH fragment and its properties show that the HDH site is carried in the native enzyme by a region of the subunit which can retain its structure when the amino terminal third of the complete polypeptide is destroyed. This justifies the name of globule which we shall give to this structure.

The main conclusions of this section are (1) the activity of the HDH fragment is insensitive to threonine; and (2) the association areas which link two globules in a dimer are not those involved in the dissociation of the native tetrameric enzyme into dimers.

The Aspartokinase Fragment

The protein of an *ochre* mutant, Gif 101,[37,38] carrying the aspartokinase activity but devoid of homoserine dehydrogenase activity, was obtained in the homogeneous state by using the classical techniques of protein purification.[39]

Stability

The enzyme is stable at 4°C in the presence of L-threonine or L-serine; K^+ ions do not stabilize the enzyme, as is found for the wild type enzyme.

Molecular Weight

The protein, in the presence of threonine (1 m*M*), has a molecular weight of 180,000[37]; it dissociates in the absence of threonine into active monomers of molecular weight 48,000.[37]

The same molecular weight of 48,000 is obtained by SDS gel electrophoresis.[33] From these data, it can be assumed that the threonine sensitive aspartokinase of Gif 101 is a tetramer made of subunits considerably shorter than those of the wild type enzyme.

Kinetic Parameters

The kinetic parameters of the tetrameric and of the monomeric aspartokinase are given in Table 5.

The aspartokinase activity of the tetrameric enzyme is threonine sensitive, and the inhibition curve displays the same homotropic cooperative effects as in the wild type enzyme.[36] The aspartokinase of the tetrameric enzyme is also sensitive to serine and homoserine.[37,39]

The monomeric form of the aspartokinase is insensitive to threonine, but retains the sensitivity to homoserine.[37]

Amino Acid Analysis and Fingerprint

The amino acid composition[39] of the carboxymethylated aspartokinase fragment is given in Table 6.

Only three of the six tryptic peptides containing cysteine found in the wild type protein are present in the mutant protein: T85, T10, and TNb (Figure 13c). It will be noted that these three peptides are not found in the HDH fragment.[33]

Localization of the Polypeptide Chain of the Aspartokinase of Gif 101 on the Complete Polypeptide

The short polypeptide chain of Gif 101 is located on the amino terminal side of the native polypeptide, as evidenced by the weak argument that the same N terminal amino acid (methionine) is found as in the wild type enzyme, but mainly by genetic evidence that the mutation is an *ochre* mutation.[38]

TABLE 6

Amino Acid Composition of the Aspartokinase Fragment

Amino acid	Residues/mol of subunit (mol wt = 48,000)
Lysine	15
Histidine	8
Arginine	25
Aspartic acid	40
Threonine	25
Serine	29
Glutamic acid	46
Proline	26
Glycine	34
Alanine	52
Valine	32
Methionine	15
Isoleucine	31
Leucine	45
Tyrosine	8
Phenylalanine	14
Tryptophan	2
Cysteine	7

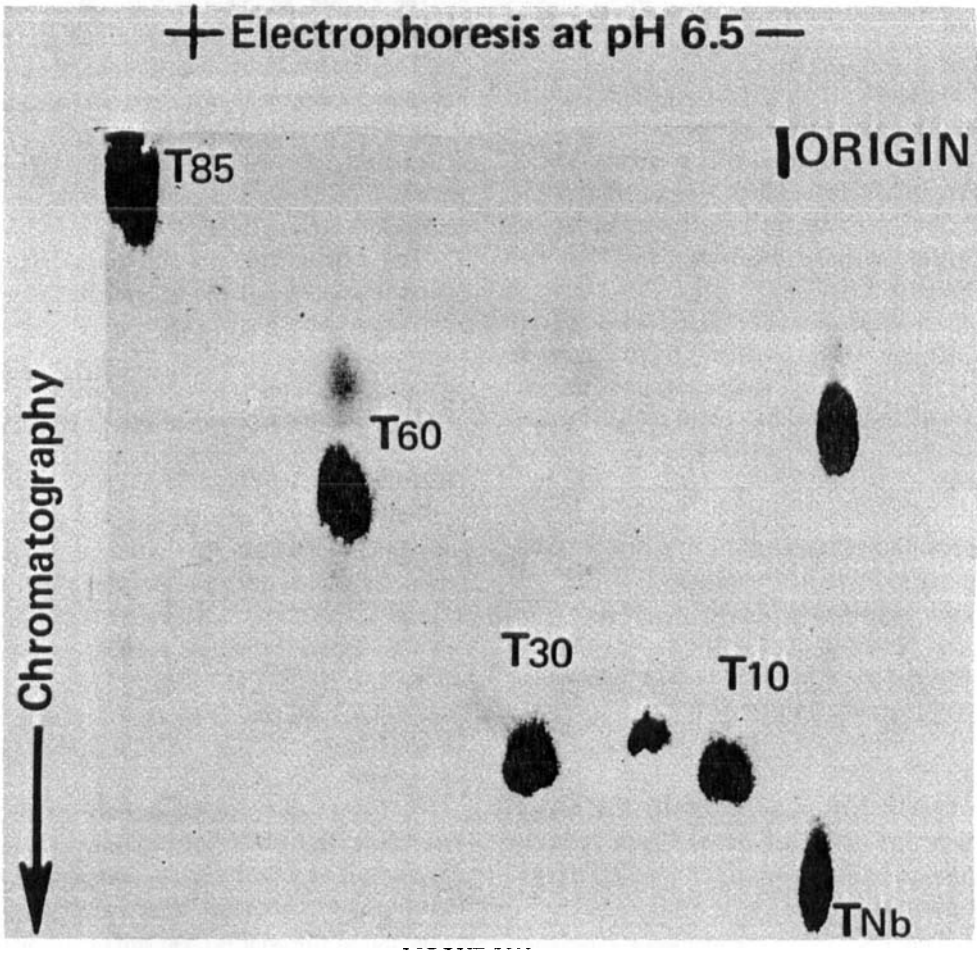


FIGURE 13. Autoradiograms of tryptic digests. (A) ^{14}C-carboxymethylated aspartokinase I-homoserine dehydrogenase I; (B) homoserine dehydrogenase fragment; (C) Gif 101 *ochre* fragment.

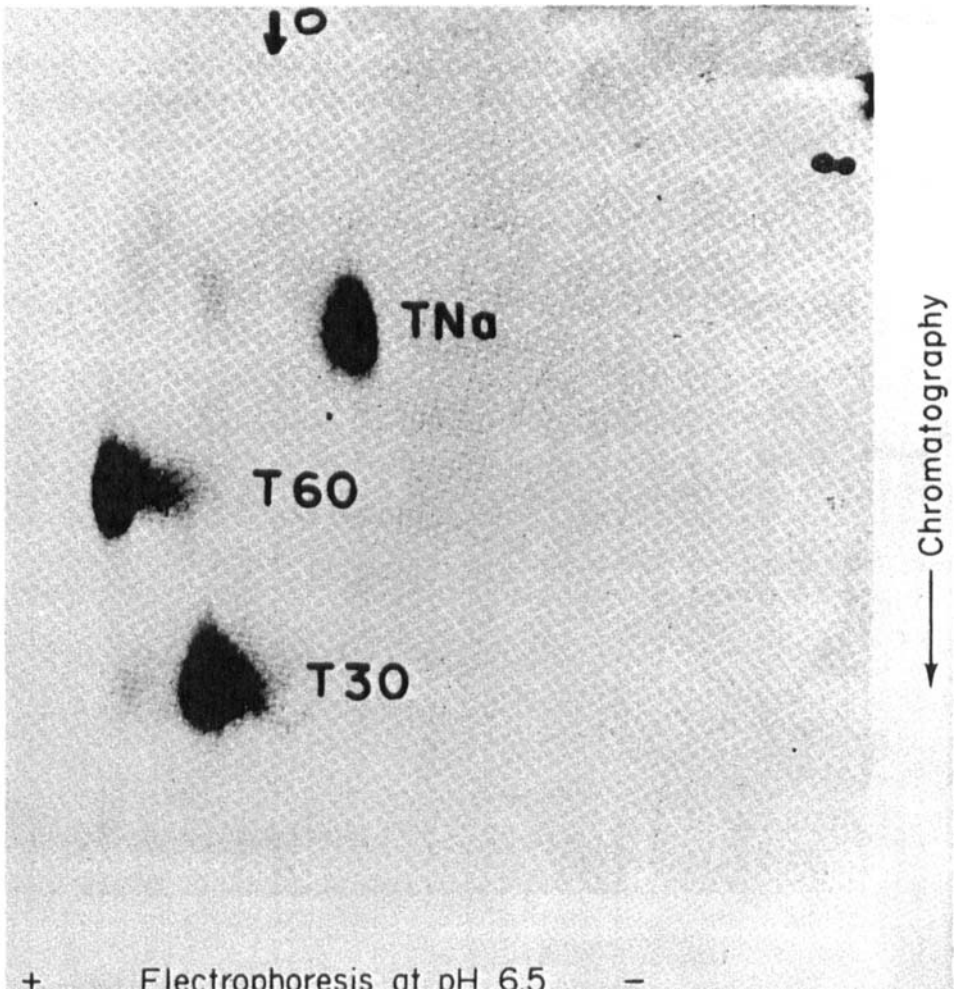


FIGURE 13B

Molecular Extinction Coefficient

The molecular extinction coefficient was calculated from quantitative analysis of amino acid content to be 21,000 ± 1,080 for a molecular weight of 48,000.[39]

Titration of the Cysteine Residues

The titration of the cysteine residues by DTNB in the absence of threonine (when the enzyme is in the monomeric form) results in the inactivation of the aspartokinase; six out of seven residues are titrated, the seventh residue being titrated upon addition of SDS.

In the presence of threonine (i.e., in the tetrameric form) only two cysteines are titrated; this titration leads to the inactivation of the aspartokinase activity. This result contrasts with the one obtained with the wild type enzyme, where threonine protects all the cysteines and, therefore, the kinase activity.[39]

Conclusion

Although they share a common, overlapping sequence over a region of about 17,000 daltons, the existence of the fragments described above proves that the two activities are carried out by distinct regions of the same polypeptide. The fact that such modified proteins with shortened subunits can be obtained, either by mutation or by mild proteolysis, gives some further indications regarding the configuration of native aspartokinase I-homoserine dehydrogenase I. They both retain, almost unchanged, one or the other of the two activities, with normal apparent affinities for the substrates. Therefore, their structure must be close to that of the corresponding region in the native

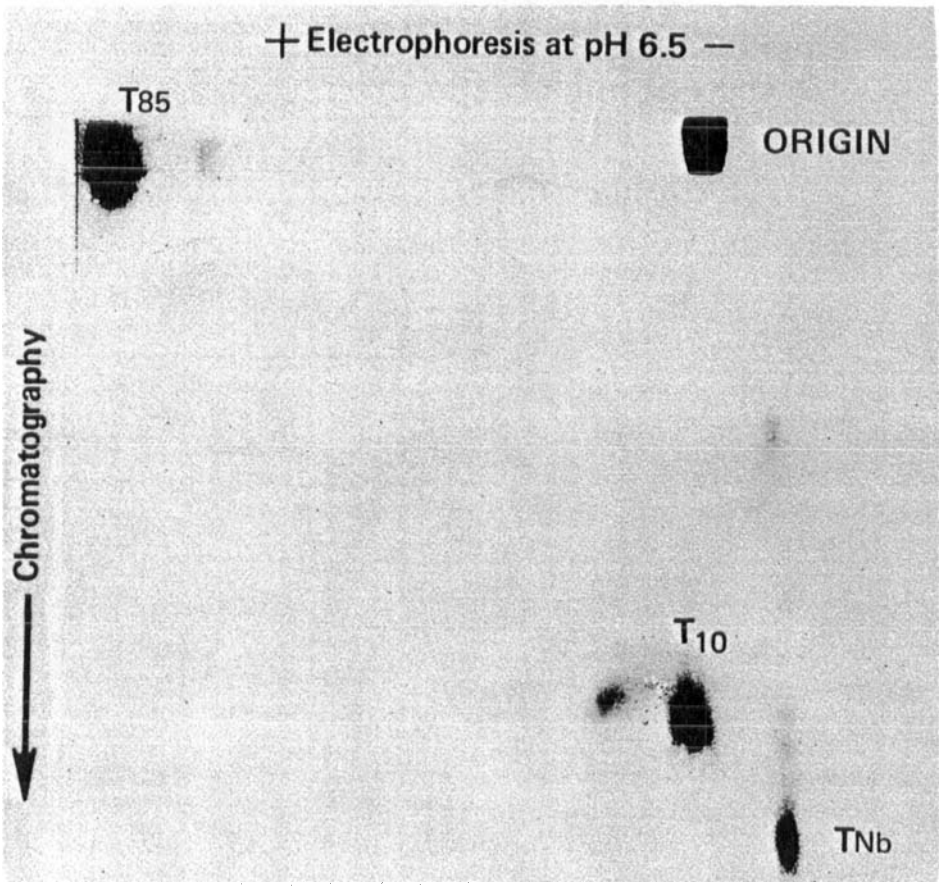


FIGURE 13C

protein. The subunit of aspartokinase I-homoserine dehydrogenase I thus appears, in its native configuration, to be composed of at least two rather independent regions or "globules", each being able to retain its own catalytic activity when the other is removed.

Attempts to achieve a functional complementation of the two fragments by mixing the desensitized HDH fragment with the sensitive AK from Gif 101 in order to recover a sensitive HDH activity have been unsuccessful; the presence of the overlap, perhaps forbids an efficient complementation. This experiment, repeated with shorter nonsense fragments, might be successful.

The proposed biglobular structure of the subunit leads to the prediction that each globule is able to achieve its folding into the correct tertiary structure in the absence of the other. This is obviously the case for the aspartokinase fragment which, being synthesized in the active form, is therefore able to fold into its tertiary structure. We were unable to obtain the renaturation of the HDH fragment after denaturation with 8 *M* urea. This is not conclusive, since no renaturation has been obtained even with the native protein.

STUDY OF THE INTERACTIONS BETWEEN THE TWO PARTS OF THE SUBUNIT

Considering that the two activities are carried out by distinct regions of the polypeptide, and that two equivalents of threonine, the allosteric inhibitor, are bound per subunit, one could imagine that each threonine site resides in one or the other of the two globules, the inhibition of the two activities thus being independent.

It is known from early experiments[40] that the substrates of one activity are uncompetitive inhibitors of the other activity, which is a first indication for the existence of interactions between the two globules. These interactions are actually of utmost importance in the inhibition process. In addition, we shall see that the two globules do not play analogous functions in the building of the tetrameric structure of the protein.

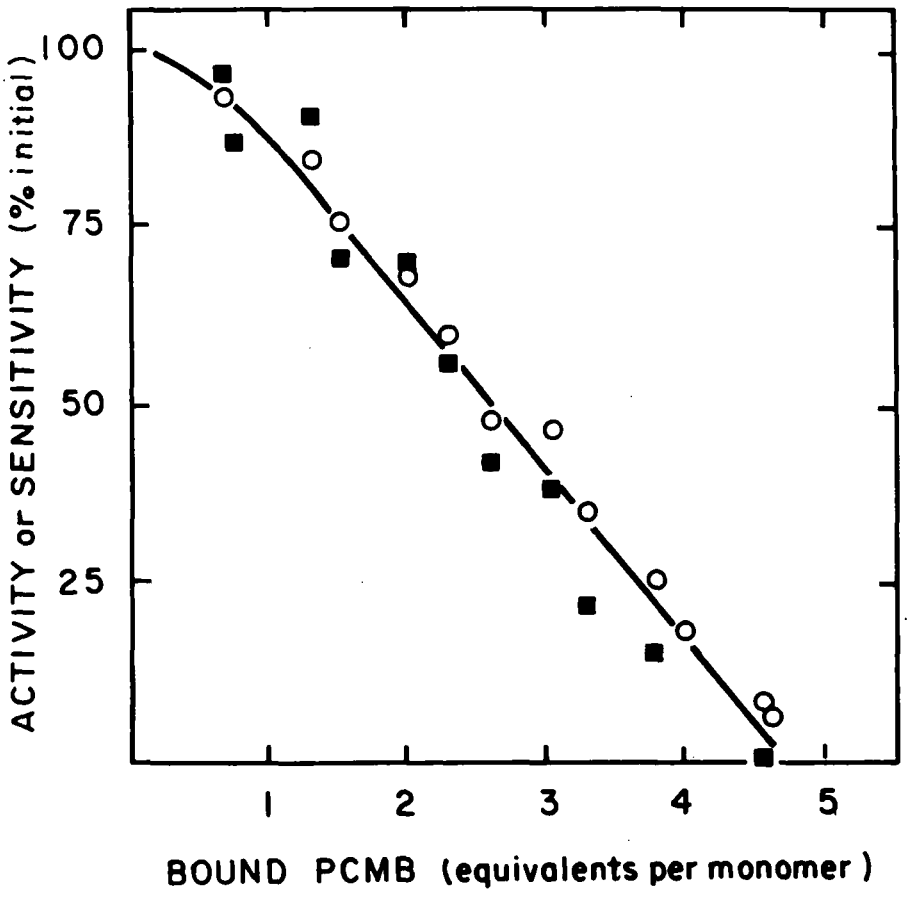


FIGURE 14. Correlation between the two catalytic activities and sulfhydryl titration by *p*-mercuribenzoate; ○: aspartokinase activity; ■: homoserine dehydrogenase sensitivity to 10 m*M* L-threonine.

Correlation between Aspartokinase (AK) Activity and Homoserine Dehydrogenase (HDH) Threonine Sensitivity

The linkage between the kinase activity and the sensitivity of the dehydrogenase activity is demonstrable under a large variety of conditions. This linkage is particularly apparent during the reaction with alkylating agents such as PMB or DTNB. Figure 14 shows a typical experiment in which the concomitant loss of AK activity and HDH sensitivity is observed. The same correlation is observed in kinetic experiments in which the two phenomena are followed as a function of the time of incubation with the reagent.[34] In our experiments, inactivation of kinase and HDH desensitization fall approximately linearly with the titration of about five -SH groups per monomer.

These results, added to the fact that the AK activity remaining at all intermediate points is fully inhibited by threonine, are consistent with an all or none mechanism for the alkylation of the -SH.[34,41]

However, in the presence of aspartate, a two phase process in the titration of -SH groups with PMB has been observed by Takahashi and Westhead.[28] As pointed out by these authors, this is probably due to the fact that aspartate, in conjunction with K^+, prevents the dissociation of the enzyme.

We believe, therefore, that under conditions where the enzyme is unable to dissociate easily, the two classes of available -SH groups observed by Takahashi and Westhead correspond, respectively, to those accessible and buried in the tetramer. On the contrary, an all or none titration of all the available sulfhydryls is observed under conditions where the enzyme dissociates into dimers.

The important point which we wish to purport is that, in all conditions where -SH titration causes the AK inactivation, the HDH sensitivity falls with

the same half-time of desensitization. This has been observed not only with PMB and DTNB, but also with NEM and ISA (see below).

The reaction of aspartokinase I-homoserine dehydrogenase I with 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate leads also to AK inactivation and HDH desensitization, and presents an interesting feature, since a single -SH group is titrated per subunit. In this case, the tetrameric structure is conserved after the reaction, showing that the reacting sulfhydryl is not involved in the association between the subunits.

It is worthwhile to note that the correlation between AK activity and HDH desensitization is not confined to -SH modification, since thermal denaturation or limited proteolysis leads to the same effect. This correlation points out the utmost importance of the interactions between the two globules, since no threonine inhibition of the HDH activity is possible in the absence of an intact kinase region.

If the inhibition of the activity of the HDH globule is controlled by the AK globule, the existence of two threonine binding sites per subunit remains unexplained. We feel unable to propose a scheme in which the necessity of the presence of these two sites would clearly appear. However, we can make some hypotheses.

One of these is to state that two threonine binding sites are carried by each AK globule; we do not have experimental data which contradict this hypothesis. In order to test it, measurements of threonine binding on the AK fragment would be necessary; such an experiment has not yet been done because of technical difficulties.

Another hypothesis is that only one of the two threonine molecules bound per subunit is involved in the mechanism of the allosteric transition. It should be recalled that the interaction coefficient (Hill number), measured either from physico-chemical studies or from inhibition data, has always been found to be between three and four showing the interaction of all the subunits. It is not possible to deduce the actual number of interacting sites in a system based only on the Hill coefficient. None the less, these data suggest that only four of the eight threonine equivalents bound are actually involved in the allosteric inhibition. In view of the structural similarity between threonine and homoserine, the possibility exists that the other four threonine equivalents bind at the homoserine dehydrogenase sites. This last hypothesis is somewhat contradicted by the unchanged serine inhibition of the HDH activity of the species rendered insensitive to 10^{-3} *M* threonine by either chemical modification or limited proteolysis.

In any case, it is important to stress that the Hill coefficient of the inhibition of the AK fragment (n = 3.6) is the same as that of the native enzyme, indicating that its allosteric properties are untouched. Thus, whatever the number of threonine equivalents involved in the allosteric transition, they are all likely to interact with the AK region. In this case, the inhibition of the HDH would follow as a consequence of the interactions between globules within the subunit.

The Kinase Region is Less Exposed in the Tetramer Than in the Dimer

Study of the Course of the Proteolytic Reaction

A priori, two schemes may be proposed: (1) the proteolytic enzyme acts on the tetramer, leading to the production of a tetrameric homoserine dehydrogenase fragment which dissociates into dimers; and (2) alternatively, the native tetramer could first dissociate to make the proteolytic attack of the resulting dimeric species possible.

As shown in Figure 15, the velocity of the proteolytic reaction is very strongly dependent on the aspartokinase I-homoserine dehydrogenase I concentration. At high concentration (approximately 10 mg/ml), the enzyme is quite resistant to the action of subtilisin; the loss of aspartokinase activity is rather small and a plateau is reached within 1 hr. It was confirmed by dodecylsulfate gel electrophoresis that only a very small proportion of the material was proteolyzed under these conditions. Upon dilution of the incubation mixture, active proteolysis occured until the kinase activity was totally destroyed.[3,4] As previously reported, the loss of kinase activity was always concomitant with the appearance of the homoserine dehydrogenase fragment band on dodecylsulfate gels. These results strongly suggest that the enzyme must first dissociate in order to be susceptible to the proteolytic attack, and that the real substrate of the protease is a dimer.

The protection against proteolysis by various ligands supports this hypothesis. Whereas KCl and threonine are antagonistic in shifting the allosteric equilibrium either to the R or the T form, these two ligands protect against proteolytic degrada-

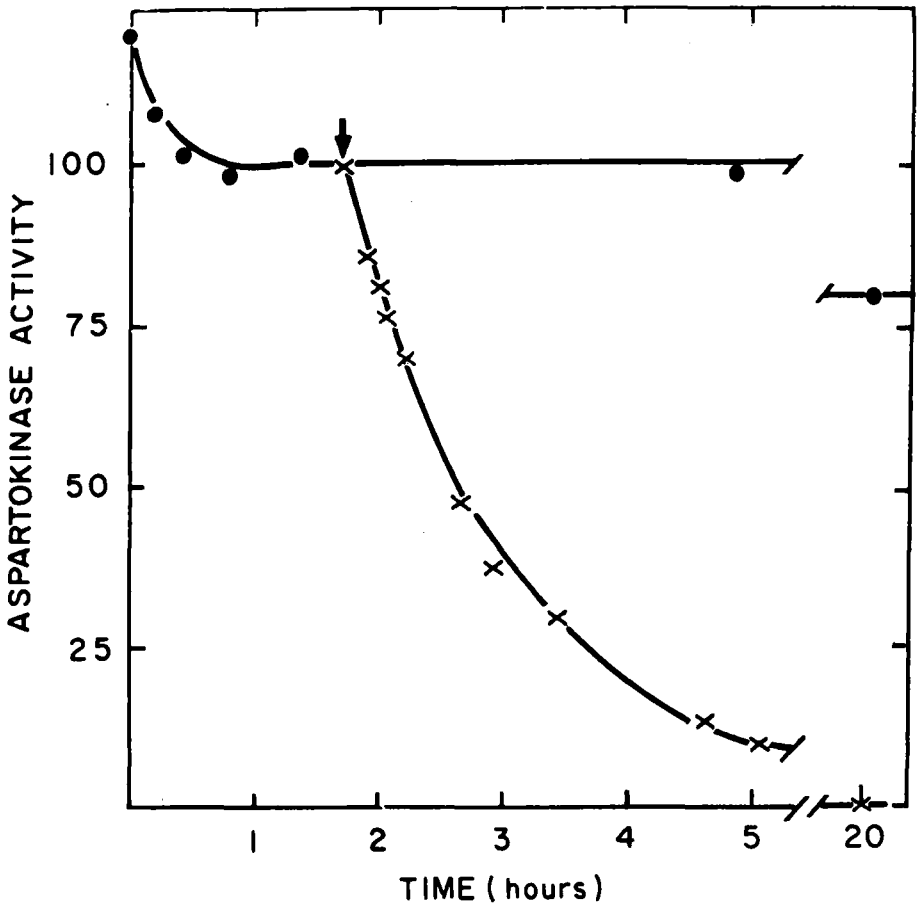


FIGURE 15. Acceleration of the rate of proteolysis of aspartokinase I-homoserine dehydrogenase I by substitution upon dilution of the incubation mixture; •: undiluted samples; X: aliquots diluted 100 times.

tion, partially for KCl and completely for threonine. This protection is well-understood in terms of the known stabilization achieved by KCl and threonine in the tetrameric state.[42] The results obtained by MacKall and Neet[35] on the proteolysis of the enzyme in a dissociating buffer (TES) are in agreement with our results. When the enzyme is in a dimeric state, the proteolysis is much faster.

Although the details of the intermediates in the proteolytic reaction are still somewhat unprecise, we can thus state with great security that the enzyme has to dissociate in a dimeric state in order to be susceptible to the proteolytic degradation which leads to the loss of the AK globule and the production of the dimeric HDH fragment. On the contrary, the AK globule is unavailable to proteolytic attack in the tetrameric state.

Study of Reactivity of Iodoacetamidosalicylic Acid (ISA)

Reaction of aspartokinase I-homoserine dehydrogenase I with ISA leads to aspartokinase inactivation and homoserine dehydrogenase desensitization, as with any -SH reagent, but it has a strong reactivity in conditions where iodoacetamide has no effect. The enzyme concentration dependance of the alkylation indicates a dissociation step in the reaction.[34] Since we have shown[34] that the salicylic moiety has a dissociation effect, we can again conclude that the aspartokinase activity is especially exposed when the enzyme dissociates to the dimer.

Immunochemical Evidence that the Aspartokinase Regions are Buried in the Native Enzyme in the Presence of Threonine

The capacity of an immunoadsorbent, prepared

TABLE 7

Immunoadsorbents Prepared Against the Wild Type and Aspartokinase Fragment Enzyme: Determination of Their Capacity According to the Presence of L-Threonine

	Anti-wild type enzyme		Antiaspartokinase fragment	
	Capacity (nanomoles of subunits)			
Protein tested	Without threonine	With threonine	Without threonine	With threonine
Wild type enzyme	1.15	1.15	0.37	0.01
Aspartokinase fragment	0.71	2.3	0.97	3.2

with antiaspartokinase I-homoserine dehydrogenase I antibodies, to retain the wild type enzyme is the same whether it is measured in the presence or absence of L-threonine. In contrast, the binding of the wild type enzyme to an immunoadsorbent made from antibodies against the aspartokinase fragment varies considerably according to the presence or absence of the allosteric ligand (Table 7). The amount of enzyme bound to this immunoadsorbent is very low in the presence of L-threonine. The enormous enhancement of the reactivity of the anti-AK antibodies against the wild type enzyme when threonine is omitted shows that in the absence of this ligand, antigenic determinants lying on the wild type protein on the kinase globule are exposed.[43]

It is interesting to note that immunoadsorbents prepared with either the anti-wild type or anti-aspartokinase fragment (Table 7) retained more aspartokinase fragment when threonine was included in the buffer. This result is not unexpected if one recalls that the tetrameric form of the Gif 101 enzyme dissociates to the monomeric state in the absence of threonine.[37] In saying this, we make the reasonable assumption that one molecule of antibody can retain either a tetramer or a monomer. Moreover, this strongly suggests that no dissociation of the tetrameric fragment occurs on binding with the antibodies, provided threonine is present.

Under the conditions of the experiment (0.15 *M* KCl and ca. 500 μg/ml protein), the enzyme in solution is in the tetramic state, whether or not threonine is present.

The capacity of an antiproteolytic fragment immunoadsorbent for the retention of native aspartokinase I-homoserine dehydrogenase I is independent of the presence of threonine.[43] Therefore, we may assume that the native enzyme reacts with immunoadsorbents in the tetrameric state, since if it dissociated without threonine, the capacity of anti-HDH fragment immunoadsorbent would be lowered by a factor of the order of two.

The experiment of mild proteolysis and the study of sulfhydryl reactivity have shown that the aspartokinase regions are more susceptible to modification when the enzyme dissociates to dimers. The proteolysis experiments were done, however, with 20 m*M* instead of 150 m*M*, K^+ and the sulfhydryl titration by ISA involves a dissociating role of the hydrophobic moiety of this reagent.[34]

These two sets of experiments prove that the kinase regions are not only accessible in the dimeric state, but that this is also probably the case in the K^+-liganded form of the tetrameric state.

Cross-linking with Dimethylsuberimidate and Mild Proteolysis of the Cross-linked Tetramer

Cross-linking of enzyme subunits with the bifunctional reagent dimethylsuberimidate yields information regarding the number of subunits in an oligomeric protein and was used to confirm the tetrameric nature of aspartokinase I-homoserine dehydrogenase I.[17]

Figure 16 illustrates the kinetics of cross-linking, as determined after gel electrophoresis. The appearance of the tetramer (band D) was parallel to the partial loss and desensitization of both activities. It should be clear in this paragraph that dimer, trimer, and tetramer refer to the observed migration on SDS gels: namely, to the state of cross-linking of the enzyme. Proteolysis of the cross-linked enzyme gave interesting indications of the nature of the association areas in the

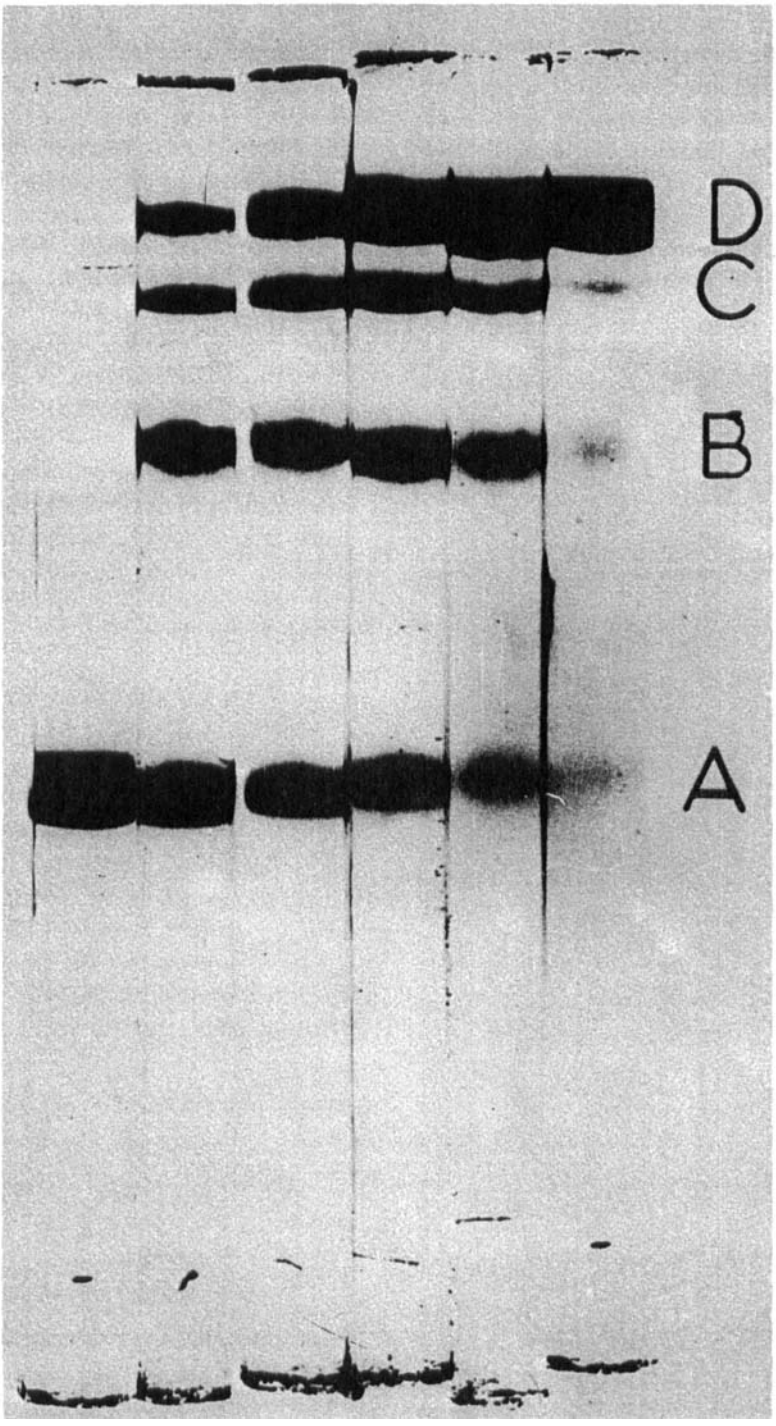


FIGURE 16. Kinetics of cross-linking of subunits: dodecylsulfate gels. Upon calibration, bands A, B, C, and D correspond, respectively, to monomer, dimer, trimer, and tetramer. From left to right, the reaction times were: 0, 2, 5, 10, 20, and 60 min.

tetramer. The cross-linked tetramer was subjected to proteolysis with α-chymotrypsin (subtilisin, in contrast to its action on the native enzyme, does not degrade the cross-linked enzyme). As shown in Figure 17, the final product of the proteolysis (band 1) has a molecular weight of 110,000, exactly that expected for the dimeric HDH fragment (2 X 55,000). The possibility that this product could be a tetrameric fragment (mol wt = 120,000) resulting from the cross-linking of the other possible product of proteolysis, namely the complementary piece of 30,000 mol wt, has been eliminated in an experiment where a partially cross-linked enzyme was proteolyzed.[34] Thus, the ultimate product after limited proteolysis with α-chymotrypsin of the fully cross-linked tetramer is a cross-linked dimeric HDH fragment: this implies that the only way to cross-link dimers into a cross-linked tetramer is by linking their aspartokinase regions together; otherwise, a cross-linked tetrameric HDH fragment of mol wt = 220,000 should have been obtained after proteolysis.

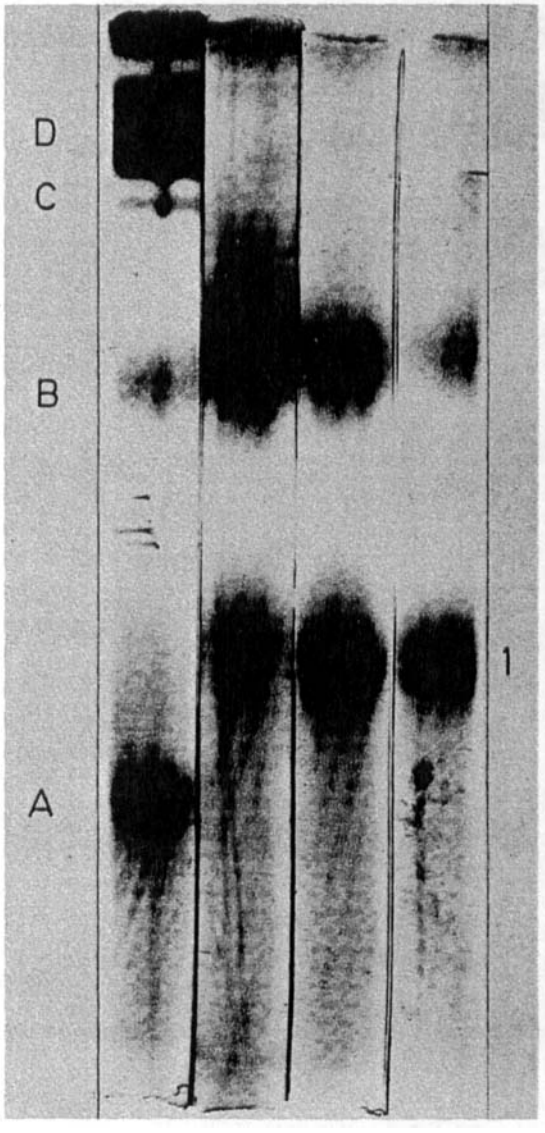


FIGURE 17. Proteolysis of the fully cross-linked tetramer. The gel on the extreme left corresponds to the mixture subjected to proteolysis (t = o); the 3 other gels correspond, respectively, to periods of proteolysis of 30 min, 2 hr, and 4 hr. Bands A, B, C, and D correspond to the same species as in Figure 16. Band 1 corresponds to a species of M_r = 110,000.

These results rule out all tetrameric assemblies based on heterologous associations. Thus, the most likely structures for the enzyme are the isologous planar and isologous tetrahedral tetramers. The existence of isologous associations was, indeed, previously suggested by the dissociability of the enzyme to the dimer.[34,35]

TENTATIVE MODEL OF ASPARTOKINASE I-HOMOSERINE DEHYDROGENASE I MOLECULE

Figure 18 shows a schematic model of the native tetramer of aspartokinase I-homoserine dehydrogenase I. This oversimplification shows the main properties we wish to stress.

The first one is that the two catalytic activities are carried by distinct regions of the polypeptide. The topological distribution of the two activities

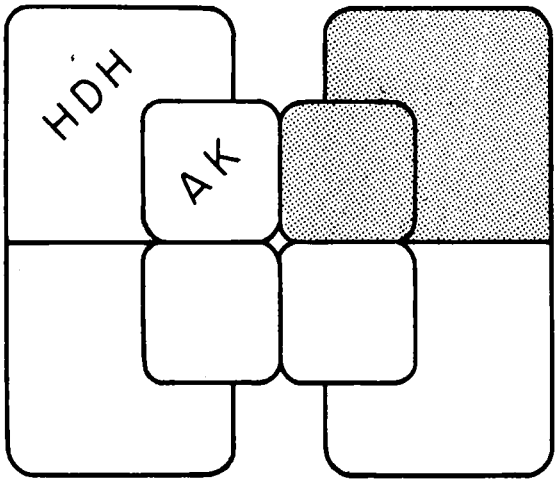


FIGURE 18. Tentative model for the organization of the subunits in aspartokinase I-homoserine dehydrogenase I.

on the subunit has been confirmed by genetic analysis of the fine structure of the gene *thrA*, the structural gene for aspartokinase I-homoserine dehydrogenase I. It has been shown that it is composed of two cistrons, with the operator proximal one ($thrA_1$) coding for the aspartokinase, whereas the operator distal ($thrA_2$) is the structural region coding for the dehydrogenase.[38]

The functional independence of the two globules shown by the isolation of active fragments is, moreover, illustrated by the properties of the missense mutants in the *thrA* gene. Among the collection of about 20 missense mutants isolated,[38] none has lost both activities, all being either of the AK^- HDH^+ or AK^+ HDH^- type, with the remaining activity being of the same order as that of the native enzyme in all the cases tested. This suggests that a disorganization of one of the globules within one subunit does not alter the catalytic activity of the other one.

The major property of the model is that the tetramer is organized in an isologous arrangement of the subunits, the aspartokinase regions forming, in a way, the core of the molecule.

This model takes into account the enhanced reactivity of chemical groups carried by the aspartokinase region in the dimeric state.

The arrangement of the homoserine dehydrogenase globules in dimers is in agreement with the structure of the proteolytic homoserine dehydrogenase fragment.

POSSIBLE ORIGIN AND RELATIONSHIP OF *ESCHERICHIA COLI* ASPARTOKINASE I-HOMOSERINE DEHYDROGENASE I, HOMOSERINE KINASE, AND OTHER ENZYMES OF THE SAME BIOCHEMICAL PATHWAY

In 1945, Horowitz[44] proposed a theory for the origin of biochemical pathways, postulating that the genes which code for the individual reactions had evolved backwards, with the first gene having appeared specifying the last enzyme of the pathway "to come", and the selective pressure for the appearance of the other genes being provided by the depletion of the end product in the original environment. The hypothesis was reiterated[45] 20 years later, with the knowledge of the structure of bacterial operons; it was rendered more plausible by the existence of allosteric inhibition, the affinity of the first enzyme of the pathway for the end product reflecting a kind of "memory" of this enzyme for its origin.

An accidental observation during the study of the purification of mutant proteins by immunoadsorption led us to investigate possible structural relationships in the enzymes involved in the threonine biosynthetic pathway, i.e., aspartokinase I-homoserine dehydrogenase I, aspartate semialdehyde dehydrogenase, homoserine kinase, and threonine synthetase. We have also investigated possible relationships between aspartokinase I-homoserine dehydrogenase I and the isofunctional enzymes aspartokinase II-homoserine dehydrogenase II and aspartokinase III.

Table 8 shows clearly that anti-AK I-HDH I immunoadsorbent retains the three isofunctional aspartokinases with varying efficiencies; it also retains *E. coli* homoserine kinase. As a control, it does not bind *E. coli* aspartate semialdehyde dehydrogenase, threonine synthetase, biosynthetic threonine deaminase, β-galactosidase, or *N*-acetylglutamokinase (the latter catalyzing a reaction similar to that of aspartokinase). Conversely, antihomoserine kinase immunoadsorbent retains aspartokinase I-homoserine dehydrogenase I.[47]

We wish to stress all the precautions which have been taken before drawing any conclusion from the above experiments. A possible contamination of the antigens used as immunogens and as reactants has been eliminated: aspartokinase I-homoserine dehydrogenase I, obtained in the pure state by conventional methods, was further filtered through an antiaspartokinase I fragment immunoadsorbent in the presence of threonine, under which conditions it is not retained[43] whereas traces of homoserine kinase would have been adsorbed. The preparation of homoserine kinase used as antigen is devoid of aspartokinase and homoserine dehydrogenase; furthermore, its N terminal sequence has been determined: the first seven amino acids come out from the automatic sequencer with no evidence of heterogeneity.[48]

These results show beyond doubt that the aspartokinase I-homoserine dehydrogenase I and the homoserine kinase from *E. coli* K12 share related antigenic structures. These structures, in aspartokinase I-homoserine dehydrogenase I, lie on the N terminal fragment, since homoserine kinase

TABLE 8

The Three Isofunctional Aspartokinases and Homoserine Kinase from *E. coli* have Antigenic Determinants in Common

Antigen	Immunoadsorbents: Antiaspartokinase I-homoserine dehydrogenase I	Immunoadsorbents: Anti-homoserine kinase
	Capacity (nanomoles of subunits)	
Aspartokinase I-homoserine dehydrogenase I	1.25	0.24
Aspartokinase II-homoserine dehydrogenase II	0.62	not done
Aspartokinase III	0.36	0.14
Homoserine kinase	0.16	2.0

is recognized by the antiaspartokinase fragment antibodies and not by the antiproteolytic fragment antibodies.[47]

The probable common origin between the two enzymes is further assessed by a homoserine dependent ATP hydrolytic activity of the homoserine dehydrogenase I-aspartokinase I (in preparation) which might be a stamp of the original ancestor of the latter. This homoserine kinase-like activity could be the structural basis for the so far difficult to explain fact of homoserine inhibition of the aspartokinase activity of the aspartokinase I-homoserine dehydrogenase I and of the aspartokinase I fragment.[37,40] It should be noted in this respect that homoserine kinase is totally devoid of aspartokinase activity.

The reactions catalyzed, although they involve similar substrates (ATP and homoserine in one case, ATP and aspartate in the other), are very different, since the products are in one case an ester and in the other a mixed anhydride. We are thus tempted to eliminate the hypothesis that the cross-reacting antibodies against aspartokinase I-homoserine dehydrogenase I and homoserine kinase are directed against antigenic determinants located at a possibly similar catalytic site, and we are left with the conclusion that the antigenic relatedness reflects a common origin.

If we then follow Horowitz's hypothesis,[44,45] we can speculate that homoserine kinase is the precursor of aspartokinase I, which might have arisen by gene duplication. In favor of this speculation, we can cite the fact that aspartokinase I-homoserine dehydrogenase I has retained a homoserine kinase-like activity and that the activity of both proteins is threonine sensitive.

How the lysine sensitive aspartokinase (AK III) and the methionine repressible aspartokinase (AK II) are related to aspartokinase I remains open to further speculation.

Whereas in *E. coli*, aspartokinase I and homoserine dehydrogenase I activities are carried by a single protein, in genera other than *Enterobacteriaceae*, the reactions are usually catalyzed by two different enzymes, each inhibited by threonine. Among others, this has been shown to be the case in *Pseudomonadaceae*, *Azotobacter*, *S. cerevisiae*, *Rhodospirillum rubrum*, and *Rh. tenue*.

A similar situation has also been encountered in the case of the different structural associations between the glutamine amino transferase activity characteristic of anthranilate synthetase and anthranilate phosphoribosyl transferase: the two activities are associated in a single polypeptide in *S. typhimurium* and *E. coli*, while they are separated in *Pseudomonas putida*, *Bacillus subtilis*, and *Serratia marcescens*. Another case is that of phosphoribosylanthranilate isomerase and indole glycerol phosphate synthetase, which exist as two separate entities in *S. cerevisiae* and as a single polypeptide chain in *E. coli*. These results, as well as the experimental fusion between two genes of the histidine operon in *S. typhimurium*, have revived the interest in an hypothesis put forward some years ago,[50] according to which gene fusion might be an important mechanism in the evolution of complex proteins. More specifically, it has been suggested[51] that a class of proteins exists whose members have evolved through gene fusion, and

that they may be found among proteins where a single long polypeptide chain carries multiple binding sites or several catalytic activities. *E. coli* aspartokinase I-homoserine dehydrogenase I certainly meets this definition, and we have proposed[33] that its structural gene has arisen by fusion. Whether the latter is a "recent" event, or whether the "nonfused" corresponding proteins derive from fused ancestors, is a question which apparently can be answered by the demonstration of the acquisition of a new property that would confer a selective advantage in one case or the other, according to the enzyme, the organism, and the ecological niche where it has evolved.

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