(*N*-ethylmorpholine and triethylenediamine) show no and little reactivity, respectively, toward I.

Added in Proof

The structures of I and derived products have been drawn with the phenyl ring cis to the ring carbonyl group. Recent X-ray and nmr data of Brocklehurst *et al.* (1971) dictate that the structures should have a trans configuration. This in no way influences the results of this study.

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Aspartokinase I–Homoserine Dehydrogenase I of *Escherichia coli* K12 λ. Subunit Molecular Weight and Nicotinamide–Adenine Dinucleotide Phosphate Binding†

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ABSTRACT: The aspartokinase I–homoserine dehydrogenase I complex isolated from *Escherichia coli* K12 was denatured in urea and sodium dodecyl sulfate and the subunits produced were examined by dodecyl sulfate–polyacrylamide gel electrophoresis and found to be homogeneous in size with a molecular weight of 85,000. The binding of $NADP^+$ by the enzyme was investigated at pH 8.79 in the presence of 0.01 M L-threonine by gel filtration. The number of $NADP^+$ binding sites per enzyme subunit was found to be 0.50 ± 0.01 and the dissociation constant for the enzyme–$NADP^+$ complex was determined to be 5.0 ± 0.2 μM. The significance of these findings to the possible structure of the native aspartokinase I–homoserine dehydrogenase I complex is discussed.

The aspartokinase I–homoserine dehydrogenase I (AKI–HSDI) complex isolated from *Escherichia coli* possesses properties which make it particularly interesting from a functional as well as a structural standpoint. A number of its properties suggest that this enzyme complex may play a key role in the control of the rate of biosynthesis of some of the

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aspartic acid family of amino acids. For example, both enzymic activities of the complex are subject to a feedback inhibition by L-threonine, one of the end products of the biosynthetic pathway, and the synthesis of the enzyme complex appears to be repressed by a combination of two of the end products of the pathway, L-isoleucine and L-threonine (Patte *et al.*, 1963). Interest in the structure of the complex stems in part from the observations made by Patte *et al.* (1966) that the two enzymic activities copurified and that the two enzymic activities were affected in a similar manner by a single mutation. These observations suggest that the enzyme complex may consist of a double-headed enzyme. At present, there appears to be general agreement among the investigators in the field that the molecular weight of the complex when determined at a high enzyme concentration and in the presence of L-threonine is 360,000 ± 30,000, as first reported by Truffa-Bachi *et al.* (1968). There is also general agreement that the enzyme complex is composed of subunits (Patte *et al.*, 1963; Barber and Bright, 1968; Cunningham *et al.*, 1968, Wampler *et al.*, 1970; Ogilvie and Sightler, 1968). Truffa-Bachi *et al.* (1969) have reported that the results of their sedimentation studies in 6 M guanidine hydrochloride indicate that the molecular weight of the subunits is 60,000 ± 2000. Thus, they have concluded that the intact complex consists of 6 subunits. On the basis of the number of peptides obtained after digestion with trypsin, they have suggested that the 6 subunits which make up the intact complex may in fact be identical. In support of the proposal that the enzyme contains six subunits, Truffa-Bachi *et al.* (1969) have reported that there are 6 N-terminal methionine residues per molecule of the complex and Janin *et al.* (1969) have reported that the complex contains 6 binding sites for L-threonine, 3 binding sites for $NADP^+$, and 3 binding sites for NADPH.

In order to investigate the subunit structure of the enzyme by a different approach, we adopted the sodium dodecyl sulfate–polyacrylamide gel electrophoretic method described by Shapiro *et al.* (1967). Weber and Osborn (1969) have found that for 40 proteins this method yields molecular weights with an accuracy of greater than ±10%. The results of our studies with the AKI–HSDI subunits are reported herein. In addition, the dissociation constant for the AKI–HSDI–$NADP^+$ complex and the number of binding sites for $NADP^+$ were determined by the method described by Hummel and Dreyer (1962) and the results of these studies are also presented.

Materials and Methods

Serum albumin (bovine), myoglobin (sperm whale), urea, Tris base, and Tris-HCl were obtained from Mann. Ovalbumin, catalase (bovine liver), phosphorylase *a* (rabbit muscle), aldolase (rabbit muscle), and pepsin were purchased from Worthington. Dithiothreitol, $NADP^+$, L-threonine (allo-free), and DL-homoserine were products of Calbiochem. Glutamate dehydrogenase was obtained from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N-N',N'*-tetramethylethylenediamine were products of Eastman. Sephadex G-25 (superfine), Sephadex G-50 (superfine), Sepharose 4B, and DEAE-Sephadex A-50 were obtained from Pharmacia. Protein concentrations in purified AKI–HSDI preparations were determined spectrophotometrically employing the value, $A_{278} = 0.46$ absorbance unit/cm² per mg, reported by Janin *et al.* (1969). The enzymatic activity was assayed as previously described (Ogilvie *et al.*, 1969).

Preparation of AKI–HSDI Complex from E. coli K12 (λ). The bacteria were grown and harvested and the enzyme complex was isolated and purified by either of the following modifications of the purification procedure previously described (Ogilvie *et al.*, 1969).

MODIFIED PROCEDURE A. The purification procedure previously described was modified by the addition of a final step consisting of chromatography on a 35 × 4.8 cm DEAE-Sephadex A-50 column. The protein sample was applied in buffer B containing 0.15 M KCl (buffer B is 0.01 M potassium phosphate, pH 7.6, containing 5 mM L-threonine, 500 μM dithiothreitol, and 500 μM EDTA) and the column was developed at room temperature with 6 l. of a linear KCl gradient, 0.15–0.50 M KCl, in buffer B. The AKI–HSDI complex was eluted as a symmetrical peak between 0.35 and 0.38 M KCl.

MODIFIED PROCEDURE B. The procedure previously described was modified by substituting chromatography on DEAE-Sephadex A-50, as described in procedure A, for the step involving chromatography on DEAE-cellulose in the original procedure.

Both procedures A and B resulted in the isolation of enzyme preparations which appeared homogeneous as judged by their behavior on polyacrylamide gel electrophoresis. Furthermore, the specific activities of these preparations were 45.5 μmoles of NADPH oxidized/min per mg of protein when assayed in the forward direction (aspartate semialdehyde to homoserine) and 3.1 μmoles of $NADP^+$ reduced/min per mg of protein when assayed in the reverse direction (homoserine to aspartate semialdehyde). The specific activity in the forward direction is identical with that reported by Truffa-Bachi *et al.* (1968). The aspartokinase activity of these preparations when assayed by the method of Black (1962) was found to be 3.8 μmoles of aspartic β-hydroxamate produced/min per mg of protein in the presence of 5×10^{-4} M L-threonine. A 97% inhibition of the aspartokinase activity was produced by 0.01 M L-threonine.

Polyacrylamide Gel Electrophoresis. The following modification of the method of Shapiro *et al.* (1967) for the dodecyl sulfate–polyacrylamide gel electrophoresis of proteins was used. All proteins were denatured in a 0.1 M sodium phosphate buffer, pH 7.2, containing 8 M urea, 0.5 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1% mercaptoethanol. The buffer was purged with nitrogen before use and denaturation was carried out at a concentration of 0.5–0.8 mg of protein/ml of buffer. Electrophoresis of 5- to 50-μg samples of the denatured proteins was performed at 50 V in 7.5% polyacrylamide columns (0.6 × 5.5 cm) prepared from a solution containing 7.5% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 0.056% ammonium persulfate, 0.04% *N,N,N',N'*-tetramethylethylenediamine, 0.1% sodium dodecyl sulfate, 0.5 M urea, and 0.1M sodium phosphate buffer, pH 7.2. After electrophoresis, the gels were stained in 7% acetic acid containing 1% Naphthol Blue Black, destained in 7% acetic acid, and scanned at 620 nm in a Gilford Model 240 spectrophotometer equipped with a 2410 Linear Transport.

$NADP^+$ Binding Equilibrium. The $NADP^+$ binding equilibrium at pH 8.79 was investigated on Sephadex G-50 columns by the method described by Hummel and Dreyer (1962). A glass column, 0.6 cm × 28.0 cm, was connected to a Zeiss MZ2D micro flow cell (1-cm light path, 0.085-ml volume) by a small polyethylene tubing. The outlet tube of the flow cell was connected by polyethylene tubing to a Holter pump which could be adjusted to give the desired flow rate (0.6–0.8 ml/10 min). The flow cell was monitored in a Zeiss PMQII spectrophotometer equipped with a recorder, an automatic

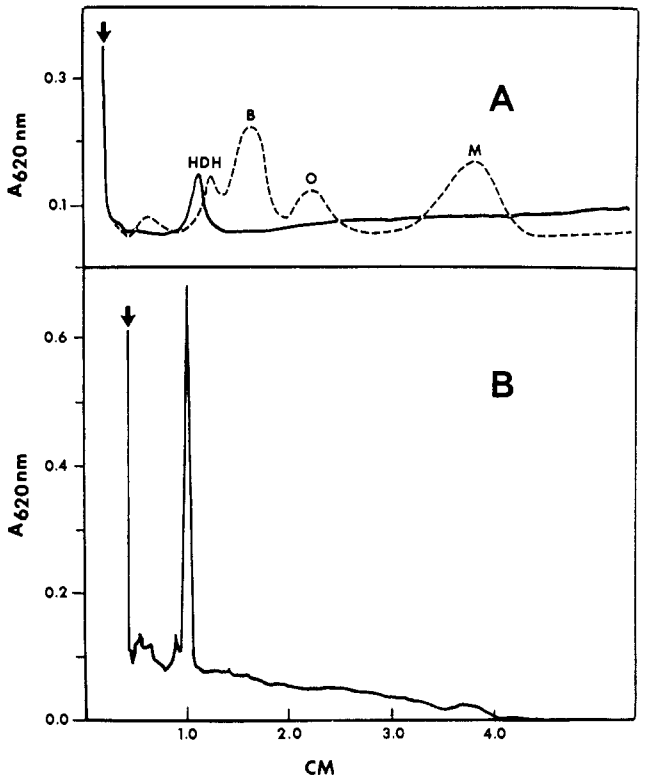

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the AKI–HSDI complex. A. Electrophoresis of approximately 5 μg of the AKI–HSDI complex in the presence (– – – –) and absence (———) of protein markers. HDH is the AKI–HSDI complex, B is bovine serum albumin, O is ovalbumin, and M is myoglobin. B. Electrophoresis of approximately 20 μg of the AKI–HSDI complex purified by procedure B. The irregular base line near top of gel is the result of damage to gel when removing it from the tube. The arrows indicate the tops of the gels.

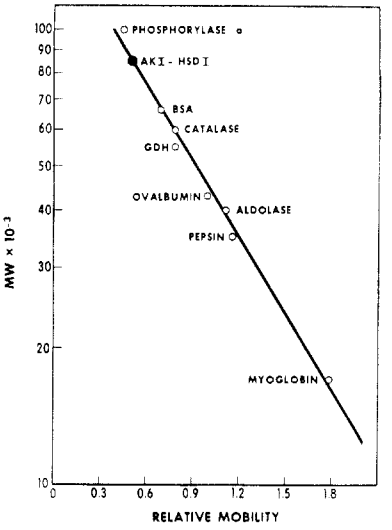

FIGURE 2: Comparison of molecular weights of eight proteins with their electrophoretic mobilities relative to ovalbumin in the sodium dodecyl sulfate–polyacrylamide gel system. Abbreviations: GDH = glutamate dehydrogenase and BSA = bovine serum albumin. The subunit molecular weight for phosphorylase *a* is that reported by Cohen *et al.* (1971). The other molecular weight values were obtained from either Klotz and Darnall (1969) or Weber and Osborn (1969).

sample changer, and an automatic zeroing device; thus, the flow cell and reference cell could be automatically switched at any desired time interval for readings and continuous rezeroing of the instrument. The column was packed to a height of 24 cm with Sephadex G-50 superfine suspended in buffer C, which consisted of 0.03 M Tris, pH 8.79, 10 mM L-threonine, 100 μM ethylenediaminetetraacetate, 100 μM dithiothreitol, and 0.1 M KCl. The column was then equilibrated with buffer C containing the desired concentration of $NADP^+$ until a stable base line was obtained when the effluent was monitored at 260 nm. The AKI–HSDI complex was dissolved in buffer C containing the same $NADP^+$ concentration used in the equilibration of the column. A 98.1-μl aliquant of the enzyme solution containing 1.4–1.8 mg of the AKI–HSDI complex was placed on the column and the column was eluted with buffer C containing the same $NADP^+$ concentration used in the sample preparation and column equilibration. The column effluent was monitored continuously at 260 nm and the amount of $NADP^+$ bound by the protein was determined by measuring the area of the $NADP^+$ depletion trough by using a planimeter as well as by carefully tracing out the depletion trough on uniform tracing paper followed by cutting out the traced area and determining its weight. The millimolar extinction coefficient for $NADP^+$ at 260 nm in the pH 8.79 buffer employed was found to be 17.9 by separate experiments.

Results

Subunit Molecular Weight Estimated by Polyacrylamide Gel Electrophoresis. Representative recorder plots of gel scans from several electrophoretic experiments in the sodium dodecyl sulfate–polyacrylamide gel system are shown in Figure 1. The scans shown in Figure 1A are for experiments employing subunits derived from the AKI–HSDI preparation purified by procedure A. The nonoverlap of the AKI–HSDI subunit peaks in the two experiments depicted is typical of the variation of migration observed in separate gel columns and is not the result of any interaction between the AKI–HSDI subunits and the protein standards which are present in one experiment and not the other. Because of this variation in migration in different experiments, all experiments were carried out in the presence of an internal protein standard, ovalbumin, and all mobilities are expressed as relative mobilities, with the mobility of ovalbumin set as 1.0. It can be seen from Figure 1A that the subunits of AKI–HSDI appear to be homogeneous, at least with respect to the size of the subunits. Figure 1B is a gel scan obtained when a larger concentration of subunits, derived from an AKI–HSDI preparation purified by procedure B, was employed. A minor peak trailing the major peak is apparent in this experiment. This minor peak was frequently observed in subunit preparations derived from AKI–HSDI preparations purified by procedure B. Since this minor peak was not observed in preparations purified by procedure A and since the amount of this minor peak present in subunits derived from preparations purified by procedure B was variable from one preparation to another, it would appear that it probably represents a contaminant not completely removed by the procedure B purification scheme. The calibration curve shown in Figure 2 was obtained by using eight marker proteins of known molecular weight. A molecular weight of 85,000 for the subunits of the AKI–HSDI complex was estimated from this curve. The relative mobilities of the protein markers and the AKI–HSDI subunits presented in Figure 2 are mean values obtained

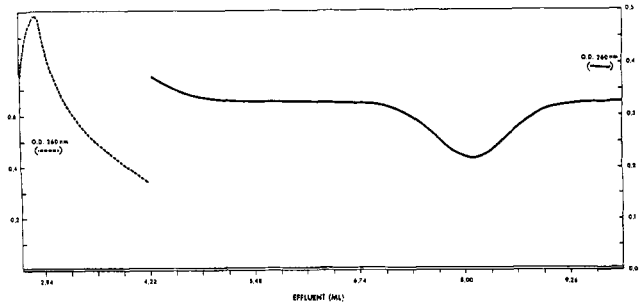

FIGURE 3: An elution profile of a gel filtration experiment in which the sample applied contained 17.10 nmoles of AKI–HSDI subunits and 2.05×10^{-5} M $NADP^+$. The column was equilibrated with a pH 8.79 buffer containing 2.05×10^{-5} M $NADP^+$ and the eluate was monitored at 260 nm.

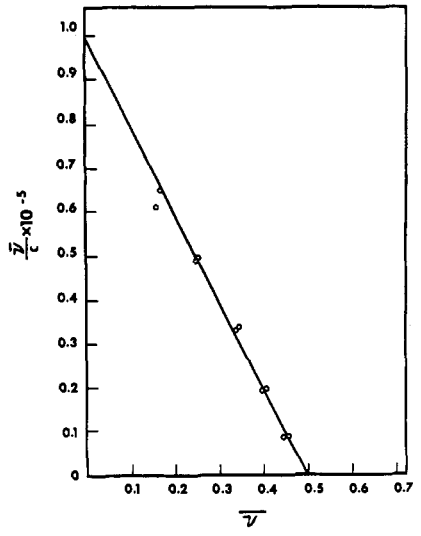

FIGURE 4: A Scatchard plot of the $NADP^+$ binding data, where $\bar{\nu}$ = average number of moles of $NADP^+$ bound per mole of enzyme subunits (subunit molecular weight = 85,000) and c = [$NADP^+$].

by averaging the relative mobilities from separate experiments. In every case, the standard error of the mean was less than 2% of the mean value.

Gel Filtration Studies of $NADP^+$ Binding. An elution profile for a representative Hummel and Dreyer type experiment is shown in Figure 3. In all experiments performed, the $NADP^+$ depletion trough was clearly separated from the enzyme–$NADP^+$ peak. A Scatchard (1949) plot of the data obtained from a series of such experiments is presented in Figure 4, where c is the concentration of free $NADP^+$, *i.e.*, the concentration of $NADP^+$ with which the column was equilibrated, and $\bar{\nu}$ is the average number of molecules of $NADP^+$ bound per subunit molecule of enzyme. The concentration of the enzyme was determined spectrophotometrically as described in the Methods section and the concentration of enzyme subunits was calculated assuming the molecular weight of a subunit to be 85,000. The values of K_{diss}, the dissociation constant for the enzyme–$NADP^+$ complex, and n, the number of $NADP^+$ binding sites per enzyme subunit, were estimated by the statistical method of Wilkinson (1961). The values found and their standard errors were $K_{diss} = 5.0 \pm 0.2$ μM and $n = 0.50 \pm 0.01$.

Discussion

Since the AKI–HSDI complex consists of subunits and possesses two enzymatic activities, it was of considerable interest to determine the molecular weight of the subunits and whether or not the subunits were homogeneous as to size. Because of its great resolving power, the sodium dodecyl sulfate–polyacrylamide gel electrophoretic method for determining polypeptide chain molecular weights seemed ideally suited for our purposes. As discussed previously, the empirical evidence of Shapiro *et al.* (1967) and Weber and Osborn (1969) constitutes strong justification for the use of this method. Furthermore, the method has been given a firm theoretical basis by the findings of Reynolds and Tanford (1970) that (a) under the conditions employed in the method, the level of binding of dodecyl sulfate to protein is high and constant on a gram to gram basis, and (b) the hydrodynamic properties of protein–dodecyl sulfate complexes are a unique function of the polypeptide chain length. All of the values for the subunit molecular weight of the AKI–HSDI complex determined in our laboratory by the gel electrophoretic method fall within the range of 85,000 ± 4000. We have recently learned that essentially identical subunit molecular weight values have been determined in two other laboratories. An AKI–HSDI preparation isolated from *E. coli* 9723 was found to consist of 80,000 ± 3000 molecular weight subunits by sedimentation equilibrium studies in guanidine hydrochloride,[1] and an AKI–HSDI preparation isolated from *E. coli* K12, mutant strain TIR-8, was found to consist of 83,000–85,000 molecular weight subunits by dodecyl sulfate–polyacrylamide gel electrophoresis.[2]

The discrepancy between our value of 85,000 for the subunit molecular weight and the value of 60,000 reported by Truffa-Bachi *et al.* (1969) on the basis of their sedimentation equilibrium studies in 6 M guanidine hydrochloride is not easily reconciled. The sedimentation coefficient of our native enzyme preparation, as determined by sedimentation studies in a sucrose density gradient in the presence of 10^{-2} M L-threonine, is 11.5S,[3] a value identical with that reported by Truffa-Bachi *et al.* (1968); the specific homoserine dehydrogenase activity of our preparation, 45.5 μmoles of NADPH oxidized/min per mg of protein, is also similar to that reported by Truffa-Bachi *et al.* (1968); and the subunits isolated from our preparation appear to be homogeneous with respect to polypeptide chain length, which is also in agreement with their results (Truffa-Bachi *et al.*, 1969). A subunit molecular weight of 85,000 would suggest that the native AKI–HSDI complex, with a molecular weight of 360,000 ± 30,000, consists of 4 subunits rather than 6 as has been proposed on the basis of a subunit molecular weight of 60,000. Since a subunit molecular weight of 80,000–85,000 has now been observed independently in three laboratories by two different techniques, it would appear that serious consideration should be given to the possibility that the native AKI–HSDI complex is indeed a tetramer. Perhaps further studies will disclose the source of the discrepancy in the subunit molecular weight.

The $NADP^+$ binding equilibrium was investigated in order to determine (a) the number of binding sites per enzyme subunit and (b) the enzyme–$NADP^+$ dissociation constant for the purpose of comparison with the dissociation constant estimated previously from kinetic studies of the enzyme

[1] Personal Communication from D. J. Cox, Department of Chemistry, University of Texas.

[2] Personal Communication from D. E. Wampler, Department of Biochemistry, University of Connecticut Health Center.

[3] Unpublished results of J. H. Sightler in this laboratory.

(Ogilvie *et al.*, 1969). The conditions employed in the binding studies were as similar as possible to those employed in the kinetic studies. Assuming a subunit molecular weight of 85,000, the number of $NADP^+$ binding sites found per subunit at pH 8.79 in the presence of 10^{-2} M L-threonine and 0.1 M KCl was 0.50. This would correspond to 2 $NADP^+$ binding sites per molecule of native enzyme if the native enzyme is a tetramer of approximately 340,000 molecular weight. This value of 2 $NADP^+$ binding sites per molecule of enzyme complex at pH 8.79 is significantly lower than the value of 3 $NADP^+$ binding sites per molecule of enzyme complex determined at pH 7.2 by Janin *et al.* (1969). The basis for this difference is unknown; however, the pH difference between the two experiments is a possible factor since a number of other properties of the enzyme complex have been observed to change over this pH range. For example, L-aspartate, a substrate for the aspartokinase activity of the complex, is an inhibitor of the homoserine dehydrogenase activity at pH 7.2 but not at pH 9.0 (Patte *et al.*, 1966).

The enzyme–$NADP^+$ dissociation constant at pH 8.79 calculated from a statistical analysis of the data of Figure 4 was found to be 5.0 ± 0.2 μM. This value for K_{diss} is approximately an order of magnitude smaller than the value (65.0 μM) previously proposed on the basis of kinetic studies and a tentative kinetic model (Ogilvie *et al.*, 1969). The explanation for this discrepancy between the values of K_{diss} obtained by the two methods is unknown; however, several obvious possibilities exist and are now being investigated. One possibility is, of course, that the tentative kinetic model previously proposed is not correct. Other possible explanations for the discrepancy are related to the fact that it was impossible to carry out the kinetic experiments and binding studies under identical conditions. For example, the enzyme concentrations in the binding studies were approximately 5000-fold higher than those employed in the kinetic studies. Since the enzyme is composed of subunits, it is possible that the enzyme dissociates into smaller units over this concentration range and that the enzyme–$NADP^+$ dissociation constant is not identical for the different states of aggregation. Although we have not thus far been able to examine the state of aggregation of the enzyme at concentrations as low as those employed in the kinetic studies, preliminary gel filtration studies at pH 8.9 in the presence of 10^{-2} M L-threonine indicate that the apparent molecular radius of the enzyme does, indeed, decrease upon dilution of the enzyme. It is also possible that the time of exposure to pH 8.79 buffer during the binding studies, which is much longer than in the kinetic studies, could be producing some alteration in the enzyme. However, if this is the case, this alteration does not appear to significantly affect the catalytic activity or the threonine sensitivity of the enzyme. Thus, it is apparent that resolution of the discrepancy in the values of the dissociation constant as determined by the kinetic and binding studies must await the results of additional studies which are now in progress.

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