Sigman, D. S., and Winer, A. D. (1970), *Biochim. Biophys. Acta 206*, 183.
Sund, H., and Theorell, H. (1963), *Enzymes 7*, 25.
Theorell, H., and McKinley McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1834.
Theorell, H., and Winer, A. D. (1959), *Arch. Biochem. Biophys. 83*, 291.
Theorell, H., and Yonetani, T. (1963), *Biochem. Z. 338*, 537.
Velick, S. F. (1961), *in* Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., The Johns Hopkins Press, p 108.
Wallenfels, K., and Sund, H. (1957), *Biochem. Z. 329*, 59.
Winer, A. D. (1958), *Acta Chem. Scand. 12*, 1695.
Winer, A. D., and Theorell, H. (1960), *Acta Chem. Scand. 14*, 1729.
Witholt, B., and Brand, L. (1968), *Rev. Sci. Instrum. 39*, 1271.
Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.

Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase of *Escherichia coli* K 12. Reaction with 6-Mercapto-9-β-D-ribofuranosylpurine 5′-Triphosphate*

Paolo Truffa-Bachi† and Henry d'A. Heck‡

ABSTRACT: An adenosine triphosphate (ATP) analog, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP), is a substrate for the aspartokinase activity of aspartokinase I–homoserine dehydrogenase I of *Escherichia coli* K 12. Incubation of the enzyme with SH-TP in the absence of L-threonine leads to a labeled enzyme of essentially the same molecular weight as the native protein which has specifically lost its aspartokinase activity while retaining in full its homoserine dehydrogenase activity. The dehydrogenase activity of the modified enzyme is desensitized to threonine, but is inhibited both by aspartate and by ATP. Reaction of the modified enzyme with mercaptoethanol releases three molecules of SH-TP per molecule of protein. Saturating levels of threonine protect the enzyme completely against SH-TP. Inactivation of the aspartokinase function by SH-TP probably does not occur by affinity labeling of the aspartokinase site(s) since (1) ATP and adenosine diphosphate (ADP) protect against SH-TP only at concentrations much greater than that of SH-TP; (2) ATP and aspartate bind to the native and modified enzymes with virtually unaltered affinities; and (3) the kinetics of reaction with SH-TP is not influenced by the presence of aspartic acid. Since reaction of the enzyme with SH-TP does not prevent binding of aspartokinase substrates, it is proposed that inactivation occurs by an interference with aspartokinase catalysis. The relevance of the SH-TP reaction to the subunit structure of the enzyme is discussed.

Physical and chemical studies of the bifunctional enzyme, aspartokinase I–homoserine dehydrogenase I (aspartokinase or ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4; homoserine dehydrogenase or L-homoserine:NADP oxidoreductase, EC 1.1.1.3) of *Escherichia coli* K 12, have shown that the protein is composed of six identical, or nearly identical, subunits (Truffa-Bachi *et al.*, 1969). The enzyme possesses six binding sites for the inhibitor, L-threonine, and the attachment of this ligand is cooperative (Janin *et al.*, 1969). On the other hand, a variety of evidence points to the existence of only three active sites for the homoserine dehydrogenase function (Janin *et al.*, 1969; Heck and Truffa-Bachi, 1970).

The recent demonstration that 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP)[1] reacts specifically with sulfhydryl groups at the adenosine triphosphatase sites of myosin (Murphy and Morales, 1970) suggested the possibility that this reagent might react selectively with the particular sulfhydryl groups necessary for the aspartokinase activity of aspartokinase I–homoserine dehydrogenase I (Truffa-Bachi *et al.*, 1966, 1968). Since the selective reaction of SH-TP with the enzyme could conceivably provide information relevant to the number of aspartokinase sites, which is a question of considerable interest, the experiments described in the present paper were undertaken.

Experimental Section

Materials. E. coli K 12, strain Tir 8 (Szentirmai *et al.*, 1968), was grown on a minimal medium containing 1% glucose as the carbon source. The cells were harvested in the late-log phase and stored at −15°. The homogeneous protein was isolated from bacterial extracts and purified as described by

* From the Departments of Bacteriology and Immunology (P. T.-B.) and Chemistry (H. d'A. H.), University of California, Berkeley, California 94720. *Received January 5, 1971.* The results of this investigation were presented in part at the 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970. This research was supported by the U. S. Public Health Service Grant No. AM 13164-02.

† Postdoctoral Fellow of the North Atlantic Treaty Organization. Present address: Service de Physiologie Microbienne, Institut Pasteur, Paris XV^e, France.

‡ Addressee for correspondence.

[1] Abbreviations used are: SH-TP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate; PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis-2-nitrobenzoate.

Truffa-Bachi *et al.* (1968). The enzyme preparation, suspended in 50% ammonium sulfate containing 2 mM L-threonine, was maintained at room temperature.

SH-TP was kindly provided by Dr. Joseph A. Duke and Miss Linda Stowring of the University of California, San Francisco. The substance was pure as evidenced by thin-layer chromatography (Murphy *et al.*, 1970).

D,L-Allylglycine was obtained from Sigma and converted into D,L-aspartyl semialdehyde according to Black and Wright (1955). L-[^{3}H]Aspartic acid was purchased from New England Nuclear. The compound had a specific activity of 2130 mCi/mmole. All other reagents and enzymes were obtained from commercial sources.

Apparatus. Absorption spectra were recorded on a Cary 14 spectrophotometer and circular dichroism spectra on a Cary 60 spectropolarimeter. Radioactive samples were counted in 10 ml of Bray's solution (Bray, 1960), using a Nuclear-Chicago liquid scintillation counter, Model Mark I. Measurements of enzymatic activity were carried out in a Zeiss PMQ II spectrophotometer.

Determination of Covalently Bound SH-TP Molecules. For reaction with SH-TP, a sample of the enzyme preparation, resuspended from ammonium sulfate, was equilibrated with the desired buffer by passing it down a 0.9 × 12 cm column of Bio-Gel P-2. The enzyme concentration was determined spectrophotometrically, using an extinction coefficient at 278 nm of 0.46 cm^2/mg (Janin *et al.*, 1969). The desired reagents, including a freshly prepared solution of SH-TP, were added to the enzyme solution, and the pH was measured.

The number of covalently bound SH-TP molecules per molecule of enzyme was determined on enzyme samples which, owing to reaction with SH-TP, had lost at least 95% of their aspartokinase activity. The reacted enzyme solution was passed down a 0.9 × 22 cm column of Bio-Gel P-10 equilibrated with 50 mM potassium phosphate–0.3 M KCl (pH 6.5). Separation of the enzyme from excess SH-TP was achieved. Tubes containing the enzyme peak were combined, and the near-ultraviolet spectrum of the reaction product was recorded. β-Mercaptoethanol was added to both sample and reference stoppered cuvets to obtain a final concentration of 80 mM. The absorbance at 322 nm due to free SH-TP increased gradually over a 1-hr period until a constant value was reached. This value, together with the protein absorbance at 278 nm, was used to calculate the number of bound SH-TP molecules per molecule of protein. The molar absorptivity of SH-TP under these conditions is 2.31 × 10^4 M^{-1} cm^{-1} at 322 nm (Hampton and Maguire, 1961).

Formation and Detection of β-Aspartyl Phosphate. The activity of the enzyme with SH-TP as substrate was tested by detection of β-aspartyl hydroxamate. The reaction mixture contained 0.2 M Tris, 0.38 M KCl, 50 mM L-[^{3}H]aspartate, 4 mM $MgSO_4$, 15 mM SH-TP, and 1.1 μM aspartokinase I–homoserine dehydrogenase I (pH 7). After incubation at room temperature for 90 min, hydroxylamine was added to a final concentration of 1.75 M in order to trap the β-aspartyl phosphate formed as β-aspartyl hydroxamate. Incubation was carried out for a further 4 hr. Control experiments in which either SH-TP was replaced by ATP (80 mM), or in which enzyme was omitted, were run in parallel.

At the end of the experiments, the mixtures were lyophilized, and the powders were resuspended in 0.5 ml of water. A 10-μl sample from each mixture was submitted to paper electrophoresis according to Manders *et al.* (1969).

Molecular Weight Determination. The molecular weight of the enzyme reacted with SH-TP was estimated by filtration on a 0.9 × 90 cm column of Sephadex G-200. A 6.5-mg sample of aspartokinase I–homoserine dehydrogenase I was incubated for 24 hr in 0.6 ml of 0.25 M potassium phosphate, 0.3 M KCl, and 3 mM $MgSO_4$ (pH 7.35), containing 6 mM SH-TP. Column markers were native aspartokinase I–homoserine dehydrogenase I, cytochrome *c*, and Blue Dextran.

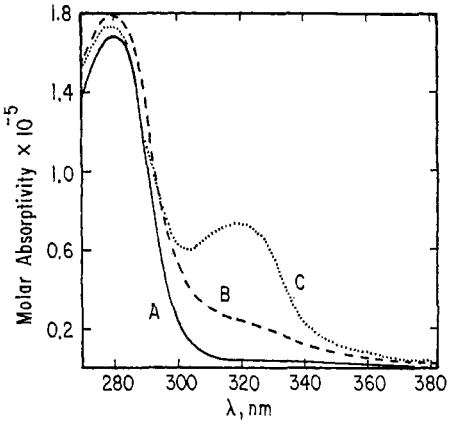

FIGURE 1: Near-ultraviolet spectra of aspartokinase I–homoserine dehydrogenase I (A); aspartokinase I–homoserine dehydrogenase I after reaction with SH-TP and separation from excess SH-TP (B); aspartokinase I–homoserine dehydrogenase I after reaction with SH-TP, separation from excess SH-TP, and addition of 80 mM β-mercaptoethanol for 1 hr (C). The spectra are recorded in 50 mM potassium phosphate–0.3 M KCl, pH 6.5, 25°.

Enzyme Assays. The aspartokinase activity was measured in a cuvet containing 0.25 M Tris, 0.5 M KCl, 12.5 mM $MgSO_4$, 0.3 mM NADH, 5.5 mM phosphoenolpyruvate, 20 mM potassium L-aspartate, 6 mM ATP, 0.3 μM pyruvate kinase, and 0.9 μM lactate dehydrogenase, at a final pH of 7.40. Homoserine dehydrogenase activity in the direction aspartyl semialdehyde to homoserine was determined in a test solution composed of 0.1 M potassium phosphate, 0.75 M KCl, 0.01 M EDTA, 2 mM L-aspartyl semialdehyde, and 0.25 mM NADPH (pH 7.31). In the reverse direction, homoserine dehydrogenase activity was tested in 0.2 M Tris, 0.5 M KCl, 35 mM L-homoserine, and 0.7 mM NADP (pH 9.0). The concentration of aspartokinase I–homoserine dehydrogenase I in all cases was of the order of 5 nM.

Results

Spectra and Stoichiometry. After reaction of aspartokinase I–homoserine dehydrogenase I with SH-TP for a time sufficient to abolish the aspartokinase activity, the near-ultraviolet spectrum of the native enzyme, Figure 1, curve A, is altered to that shown in Figure 1, curve B. The reaction product (hereafter aspartokinase–SS-TP) can be reacted further with β-mercaptoethanol to yield the spectrum shown in Figure 1, curve C. The spectra presented in Figure 1 are very similar to those observed by Murphy and Morales (1970) in the myosin system.

The shoulder at 320 nm in the absorption spectrum of aspartokinase–SS-TP was found to be optically inactive in 50 mM potassium phosphate–0.3 M KCl (pH 6.5). No extrinsic dichroism band is observed in this spectral region, either in the presence or in the absence of 10 mM $MgCl_2$.

Experiments designed to yield the number of SH-TP molecules covalently bound per molecule of enzyme are summarized in Table I. This table shows that under a variety of experimental conditions, including various times of incubation,

TABLE I: Covalent Bonding of SH-TP to Aspartokinase I–Homoserine Dehydrogenase I of *E. coli* K 12.

Time of Incubn (hr)[a]	Buffer[b]	pH	Concentration of Enzyme (μM)	Concentration of SH-TP (mM)	10 mM Potassium L-Aspartate	No. of SH-TP Bound/Molecule of Enzyme
12	Phosphate	7.2	2.8	1.5	+	3.0
20	Phosphate	7.2	2.8	2.4	+	3.2
26	Phosphate	7.3	8.7	4.4	−	3.0
16	Tris	7.8	8.3	1.7	+	2.7

[a] At 25°. [b] Phosphate buffer is 20 mM potassium phosphate, 0.3 M KCl, and 3 mM $MgSO_4$. Tris buffer is 50 mM Tris, 0.3 M KCl, and 30 mM $MgSO_4$.

different buffers, and both the presence and absence of aspartic acid, the number of covalently bound SH-TP molecules is consistently 3 ± 0.3. (An early experiment suggested the possibility that aspartic acid may influence the kinetics of reaction with SH-TP. More careful studies showed, however, that this was not the case, see below.)

SH-TP as a Substrate for Aspartokinase I–Homoserine Dehydrogenase I. Paper electrophoresis of the incubation mixtures described earlier produced the results shown in Figure 2. When enzyme is omitted, only aspartic acid can be detected, which travels approximately 20 cm in 4 hr under the experimental conditions (Figure 2, curve C). If SH-TP is replaced by ATP, and enzyme is included, two spots are observed, one appearing in the aspartic acid region, the other remaining near the origin (Figure 2, curve B). In accordance with Manders *et al.* (1969), we attribute the latter spot to β-aspartyl hydroxamate. When SH-TP is the substrate, two spots are again observed, as shown in Figure 2, curve A. The maxima correspond well to the above noted compounds.

Molecular Weight of Aspartokinase-SS-TP. The product of reaction of SH-TP with aspartokinase I–homoserine dehydrogenase I, when submitted to gel filtration on Sephadex G-200, emerges in the same elution volume as the native enzyme, as monitored both by protein absorbance at 280 nm and by homoserine dehydrogenase activity tests. On this basis, therefore, the molecular weights of the two materials are essentially identical.

Kinetics of Inactivation. The disappearance of aspartokinase activity during reaction of aspartokinase I–homoserine dehydrogenase I with excess SH-TP is a cleanly pseudo-first-order process to at least 80% of reaction, as shown by the filled triangles of Figure 3B. During the same time period, the same enzyme sample suffers no loss in homoserine dehydrogenase activity, as illustrated by the filled circles of Figure 3A.

Although the dehydrogenase activity is unaltered by reaction of the enzyme with SH-TP, the ability of L-threonine to *inhibit* the dehydrogenase activity, *i.e.*, the sensitivity of the dehydrogenase to threonine, is lost. This is shown by the open circles of Figure 3A. For these measurements, small samples of the incubation mixture were diluted, at the times shown, into dehydrogenase assay solutions which contained

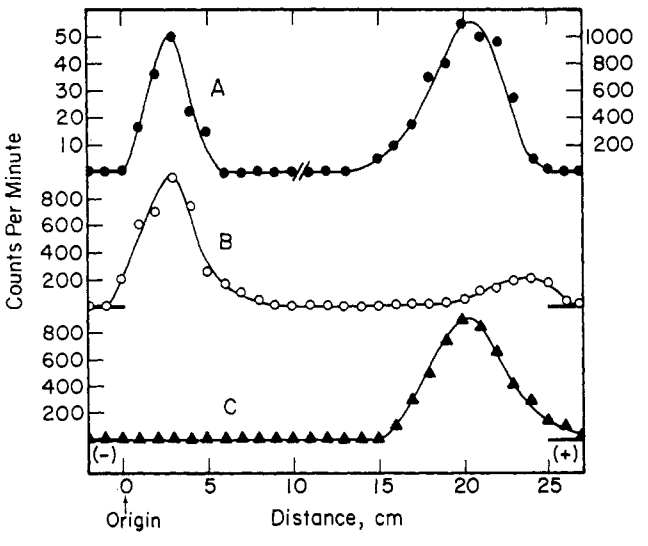

FIGURE 2: Electrophoretic identification of β-aspartyl hydroxamate and aspartate. Aliquots were subjected to electrophoresis at 30 V/cm for 4 hr on Whatman No. 1 paper at pH 6.4 in pyridine–glacial acetic acid–H_2O (400:7:1800, v/v). Aspartyl hydroxamate remains near the origin.

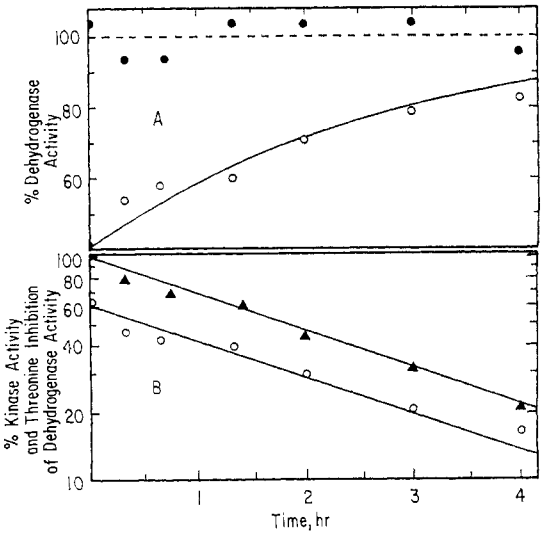

FIGURE 3: Time course of reaction of SH-TP with aspartokinase I–homoserine dehydrogenase I at 25°. The solution contains 0.7 M potassium phosphate, 0.12 M KCl, 1.2 mM $MgSO_4$, 6.7 mM SH-TP, and 4.5 μM aspartokinase I–homoserine dehydrogenase I (pH 7.40). (A) Homoserine dehydrogenase activity in the direction homoserine to aspartyl semialdehyde (●); homoserine dehydrogenase activity in the same direction in presence of 25 mM L-threonine (○). (B) Aspartokinase activity (▲); threonine inhibition of homoserine dehydrogenase activity (difference between open circles and dashed line of Figure 3A) (○).

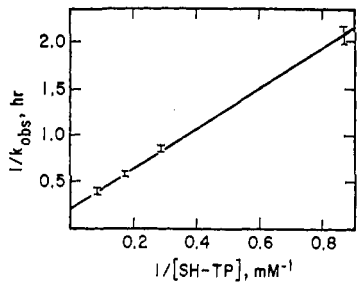

FIGURE 4: Plot of the inverse of the observed rate constant for aspartokinase inactivation by SH-TP at 25° *vs.* the inverse of the concentration of SH-TP. The solutions contained 0.7 M potassium phosphate, 0.12 M KCl, 1.2 μM aspartokinase I–homoserine dehydrogenase I (pH 7.58), and various concentrations of SH-TP as shown.

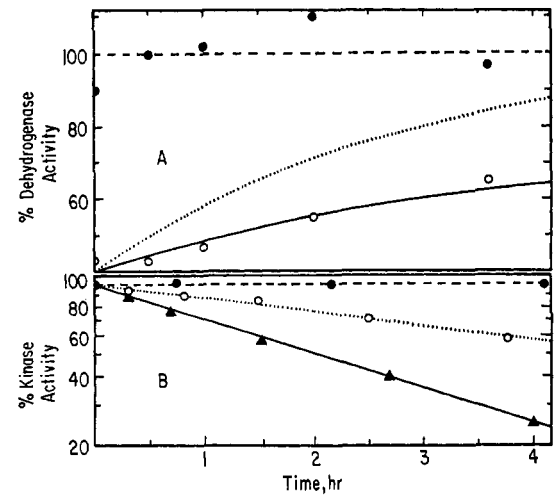

FIGURE 5: Time course of reaction of SH-TP with aspartokinase I–homoserine dehydrogenase I in the presence of ATP, ADP, and L-threonine at 25°. (A) Solution is the same as Figure 3 except that it contains additionally 0.33 M ADP. Homoserine dehydrogenase activity in the direction homoserine to aspartyl semialdehyde (●); homoserine dehydrogenase activity in the same direction in presence of 25 mM L-threonine (○); homoserine dehydrogenase activity in the same direction in the presence of 25 mM L-threonine if solution does *not* contain ADP (taken from Figure 3A) (dotted line). (B) Aspartokinase activity in a solution composed of 1.14 M potassium phosphate, 48 mM KCl, 0.48 mM $MgSO_4$, 8.8 mM SH-TP, and 1.8 μM aspartokinase I–homoserine dehydrogenase I, pH 7.42 (▲); aspartokinase activity in the same solution except for the presence of 0.33 M ATP (○); aspartokinase activity in a solution composed of 0.53 M potassium phosphate, 43 mM KCl, 0.43 mM $MgSO_4$, 9.6 mM SH-TP, 1.6 μM aspartokinase I–homoserine dehydrogenase I, and 14 mM L-threonine, pH 7.35 (●).

25 mM L-threonine. It is clear that, as reaction with SH-TP proceeds, threonine becomes progressively less effective as an inhibitor. The dehydrogenase activity, measured in the presence of threonine, approaches asymptotically the value observed in its absence.

The per cent of inhibition by threonine, *i.e.*, the difference between the open circles and dashed line of Figure 3A, when plotted in semilogarithmic form, yields the open circles of Figure 3B. It is apparent that the desensitization toward threonine proceeds at the same velocity as the loss of aspartokinase activity. The solid curve of Figure 3A is the calculated curve based on the rate constant of 0.40 hr^{-1} obtained from the plots of Figure 3B.

The order of the reaction with respect to SH-TP was determined by measuring the dependence of the pseudo-first-order rate constant for aspartokinase inactivation on SH-TP concentration. The observed rate constant did not increase linearly with SH-TP, but approached a limiting value with increasing concentration of the inactivator. The results may be analyzed in terms of the mechanism

$$\text{AK} + \text{SH-TP} \overset{K_S}{\rightleftharpoons} \text{AK–SH-TP} \xrightarrow{k} \text{AK–SS-TP} \qquad (1)$$

in which AK represents free enzyme, AK–SH-TP is an enzyme–SH-TP complex, and AK–SS-TP is the product of a covalent reaction occurring within the complex. For this mechanism, the apparent inactivation rate constant is given by

$$k_{obsd} = \frac{k}{1 + K_S/[\text{SH-TP}]} \qquad (2)$$

so that a plot of the inverse of k_{obsd} should be proportional to the inverse of the SH-TP concentration. Such a plot is shown in Figure 4. From the slope and intercept of this figure, the quantities $k = 4.5 \pm 0.7$ hr^{-1} and $K_S = 9 \pm 2$ mM are obtained.

The rate of reaction of SH-TP with aspartokinase I–homoserine dehydrogenase I is not sensitive to the presence of L-aspartic acid at two different values of pH. Thus, in 0.63 M potassium phosphate, 0.15 M KCl, 1.5 mM $MgSO_4$, 8 mM SH-TP, 5.7 μM aspartokinase I–homoserine dehydrogenase I (pH 7.40 and 7.82), and 50 mM potassium L-aspartate, the rates of aspartokinase inactivation were identical with those observed in control experiments which were otherwise the same but contained no aspartic acid. On the other hand, under similar reaction conditions, 14 mM L-threonine protects completely the aspartokinase activity against inactivation by SH-TP (Figure 5B, dashed line). The latter experiments were made possible by 350-fold dilutions of samples from the SH-TP incubation mixture into the aspartokinase test solutions, which brought the threonine concentration to the noninhibitory level of 40 μM.

Protection of the Aspartokinase Activity against SH-TP by Adenosine Di- and Triphosphates. In the presence of the coenzymes, ATP and ADP, the rate of reaction of SH-TP with the enzyme is decreased. Thus, by comparing the full and dotted lines of Figure 5B, it can be seen that the rate of disappearance of aspartokinase activity in the presence of 8.8 mM SH-TP is lowered by 0.33 M ATP, but that inactivation still occurs, even at this high ATP to SH-TP molar ratio.

The coupled aspartokinase assay used in our experiments precluded a direct measure of the protective effect of ADP against aspartokinase inactivation. It was, however, possible to demonstrate that ADP decreases the rate at which the dehydrogenase activity is desensitized toward threonine. As shown in the dashed line of Figure 5A, incubation of the enzyme with 6.7 mM SH-TP in the presence of 0.33 M ADP does not lead to a measurable loss in the homoserine dehydrogenase activity. If, however, 25 mM L-threonine is present in the dehydrogenase assay solutions, it can be seen that the rate of threonine desensitization by SH-TP in the presence of this concentration of ADP, shown by the open circles of Figure 5A, is less than one-half that observed in the absence of ADP under the same reaction conditions (Figure 5A, dotted line). Thus, ADP at these concentrations partially protects the enzyme against threonine desensitization by SH-TP.

The protection provided by ATP against SH-TP inactiva-

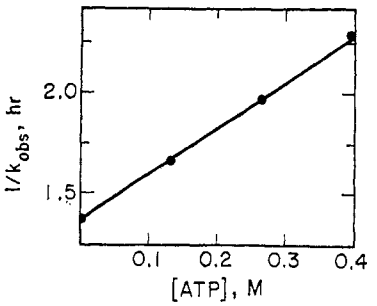

FIGURE 6: Plot of the inverse of the observed rate constant for aspartokinase inactivation by SH-TP at 25° *vs.* concentration of ATP. The solutions contain 0.79 M potassium phosphate, 83 mM KCl, 0.83 mM $MgSO_4$, 4.6 mM SH-TP, 1.1 μM aspartokinase I–homoserine dehydrogenase I (pH 7.60), and various concentrations of ATP as shown.

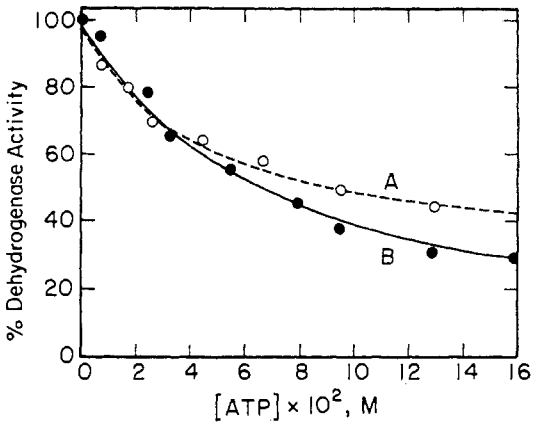

FIGURE 7: Inhibition of the homoserine dehydrogenase activity of native enzyme (A) and of aspartokinase–SS-TP (B) by ATP in the homoserine to aspartyl semialdehyde dehydrogenase test solution (see Experimental Section).

tion can be used to estimate the apparent dissociation constant of ATP from the SH-TP reaction site. If it is assumed that a site to which ATP is bound cannot react with SH-TP, the reaction may be written as

$$\begin{array}{l} \text{ATP} \\ + \\ \text{AK} + \text{SH-TP} \overset{K_S}{\rightleftharpoons} \text{AK–SH-TP} \xrightarrow{k} \text{AK–SS-TP} \\ K_A \uparrow\downarrow \\ \text{AK–ATP} \end{array} \qquad (3)$$

For this case, a simple derivation leads to the expression

$$k_{obsd} = \frac{k}{1 + \dfrac{K_S}{[\text{SH-TP}]}\left(1 + \dfrac{[\text{ATP}]}{K_A}\right)} \qquad (4)$$

From eq 4, at an essentially constant SH-TP concentration, the inverse of k_{obsd} should be proportional to the concentration of ATP. The apparent dissociation constant, K_A, for ATP from the SH-TP binding site can be calculated from the slope and intercept of such a plot, since K_S is known. The plot is shown in Figure 6. It may be calculated from this figure that K_A = 0.4 M, *i.e.*, the ATP dissociation constant is approximately 44 times greater than that of SH-TP.

Although it has been stated that aspartate does not influence the kinetics of SH-TP reaction, the possibility that aspartate might influence the extent of ATP protection against SH-TP was tested. Two solutions were prepared containing 0.65 M potassium phosphate, 0.14 M KCl, 0.136 M ATP, 2.8 mM SH-TP, and 0.9 μM aspartokinase I–homoserine dehydrogenase I (pH 7.53). One of the solutions contained 46 mM potassium L-aspartate. The rate of aspartokinase inactivation was identical in each case. Thus, no effect of aspartate on ATP protection was observed.

Inhibition of the Dehydrogenase Activity by Adenosine Di- and Triphosphates and by Aspartate. It was established by Patte *et al.* (1966) that the substrates for the aspartokinase activity are, to greater or lesser extents, inhibitors of the dehydrogenase activity, and, similarly, that the dehydrogenase substrates are weak inhibitors of the aspartokinase activity. In view of the fact that reaction with SH-TP fails to cause a decrease in the dehydrogenase activity, and of the observation of a large difference between the dissociation constants for ATP and SH-TP from the SH-TP reaction site, it seemed important to investigate the sensitivity of the dehydrogenase activity to ATP both before and after reaction with SH-TP.

Illustrations of these inhibitions are shown in Figure 7. It is seen in curve A of this figure that the homoserine dehydrogenase activity of the native enzyme decreases with increasing concentrations of ATP, as expected (Patte *et al.*, 1966). The dashed line is the curve calculated for an ATP dissociation constant K_A = 0.04 M, and a maximal inhibition at infinite ATP concentration of 72%. Curve B of Figure 7 shows that the homoserine dehydrogenase activity of aspartokinase–SS-TP also decreases with increasing concentrations of ATP. The full line is the calculated curve based on an ATP dissociation constant K_A = 0.065 M, and a maximal ATP inhibition of 100%. Thus, at sufficiently high levels of ATP, the dehydrogenase activity of aspartokinase–SS-TP is more sensitive than that of the native enzyme to ATP. The explanation for the difference between the dissociation constant for ATP from the native enzyme calculated from these experiments, and the tenfold larger value calculated from ATP protection against SH-TP (see above) may lie, at least in part, in the different buffer compositions and values of pH in the two sets of experiments.

At a fixed, high concentration of ATP, the increasing sensitivity of the dehydrogenase to ATP arising from reaction with SH-TP was followed as a function of time. The incubation mixture was composed of 0.7 M potassium phosphate, 0.12 M KCl, 1.2 mM $MgSO_4$, 8.8 mM SH-TP, and 0.7 μM aspartokinase I–homoserine dehydrogenase I, pH 7.37, 25°. Dehydrogenase test solutions contained 80 mM ATP. The rate at which the sensitivity of the dehydrogenase to ATP increased was found to be identical with the rate at which aspartokinase activity was lost, up to 95% inactivation of the aspartokinase activity by SH-TP. This result suggests that the same rate-limiting step is involved in each event.

The possibility that ADP might also be able to inhibit the dehydrogenase activity of aspartokinase–SS-TP was tested in a single experiment. The dehydrogenase assay solution for the direction homoserine to aspartyl semialdehyde was employed, and contained, in addition to the usual components, 0.134 M ADP. A control experiment was run which did not contain ADP. The dehydrogenase activity of aspartokinase–SS-TP in the presence of this concentration of ADP was 57% of the control value, showing that ADP also inhibits the dehydrogenase activity of aspartokinase–SS-TP.

The sensitivity of the dehydrogenase activity to aspartate in the native enzyme and in aspartokinase–SS-TP was tested in the direction aspartyl semialdehyde to homoserine. The results are shown in Figure 8. It is clear that the dehydrogenase activities of both enzymes are sensitive to aspartate, and that there is little difference between the sensitivities of the native and modified enzymes with respect to this compound. The curve was calculated assuming an aspartate dissociation constant of 20 mM, and a maximal inhibition at infinite aspartate concentration of 90%. A very similar dissociation constant for aspartate may be estimated from the data of Patte *et al.* (1966) and of Truffa-Bachi *et al.* (1966). The inhibition of the dehydrogenase by aspartate has been described as noncompetitive (Patte *et al.*, 1966). In agreement with this description is the present observation that the maximal degree of inhibition by aspartate is approximately 90%, indicating that aspartate probably does not bind to the aspartyl semialdehyde site.

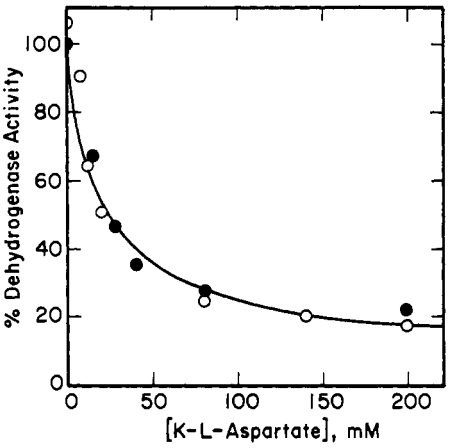

FIGURE 8: Inhibition of the homoserine dehydrogenase activity of native enzyme (○) and of aspartokinase-SS-TP (●) by aspartate in the aspartyl semialdehyde to homoserine dehydrogenase test solution (see Experimental Section).

Discussion

Evidence that SH-TP Forms a Mixed-Disulfide Bond with the Protein. That SH-TP reacts with sulfhydryl groups on the protein seems clear from several points of view. First, the spectra show that the product of reaction with SH-TP can itself be reacted with β-mercaptoethanol to liberate SH-TP. Second, reaction of the enzyme with SH-TP leads to desensitization of the homoserine dehydrogenase activity to threonine, and to an increased sensitivity of this activity to ATP. The same effects are observed when the enzyme is reacted with two sulfhydryl reagents, *p*-mercuribenzoate (PMB) and 5,5′-dithiobis-2-nitrobenzoate (DTNB) (Truffa-Bachi *et al.*, 1966, 1968). (These reagents are, however, much less selective in their reactivity than is SH-TP, see below.) Third, threonine protects the enzyme completely against SH-TP. Correspondingly, threonine protects against PMB and DTNB (Truffa-Bachi *et al.*, 1966, 1968).

Specificity of the Reaction with SH-TP. The evidence presented in this paper shows that, in the reaction with aspartokinase I–homoserine dehydrogenase I, SH-TP exhibits a high degree of selectivity when compared to ordinary sulfhydryl reagents. As indicated in Table I, SH-TP reacts under a variety of conditions with only 3 sulfhydryl groups/enzyme molecule. In contrast, the reaction of the enzyme with other sulfhydryl reagents, such as PMB or DTNB, is a complex process, occurring rapidly at 16–18 sites/360,000 daltons, and more slowly at a further 10–12 sites (Truffa-Bachi *et al.*, 1966, 1968).

The specificity displayed by SH-TP in its reaction with aspartokinase I–homoserine dehydrogenase I may be explained on the basis of the mechanism shown in eq 1. The kinetics suggest that aspartokinase inactivation occurs through the formation of an intramolecular disulfide bond in an enzyme–SH-TP complex, rather than through a bimolecular reaction involving SH-TP and an exposed sulfhydryl group on the protein. Thus, just as in ordinary enzyme-catalyzed processes, the specificity of the inactivation reaction is largely a result of an initial binding step.

The selectivity of SH-TP reaction adequately explains both the cleanly pseudo-first-order kinetics of aspartokinase inactivation and the constancy throughout reaction of both the homoserine dehydrogenase activity and the protein molecular weight. In contrast to these results, when nonselective sulfhydryl reagents such as PMB or DTNB are employed, complex kinetics are observed, owing to reaction at numerous sites (Truffa-Bachi *et al.*, 1966, 1968), and both inactivation of the dehydrogenase activity and dissociation of the protein take place following extensive reaction with PMB (Truffa-Bachi, 1968).

Mechanism of Aspartokinase Inactivation by SH-TP. Although SH-TP is both a substrate for the aspartokinase activity and a specific inactivator of this activity, three kinds of evidence suggest that covalent attachment of this molecule to the enzyme does not take place at the aspartokinase site(s). First, the normal substrates, ATP and ADP, are incapable of protecting the aspartokinase activity against SH-TP except when present at extremely high concentrations. Second, the affinity of the enzyme for ATP, as measured by ATP inhibition of the dehydrogenase activity, is essentially unaltered after the enzyme has been reacted with SH-TP. Third, the kinetics of aspartokinase inactivation by SH-TP is not affected by the presence of aspartate, whereas this substrate is known to influence the binding of ADP (J. Janin, personal communication).

None of these kinds of evidence entirely excludes the possibility that SH-TP reaction occurs at the aspartokinase site(s). Nevertheless, it would seem more prudent to conclude from the totality of data that SH-TP reaction takes place elsewhere on the enzyme molecule. How, then, does inactivation of the kinase occur?

Three possible mechanisms could account for the effects of SH-TP on the aspartokinase activity. Covalent reaction with SH-TP could (1) prevent ATP from binding, (2) prevent aspartate from binding, or (3) interfere with aspartokinase catalysis. The first two possibilities would appear to be excluded by the data of Figures 7 and 8; there is little difference between the native and modified enzymes with respect to the sensitivities of their dehydrogenase activities to aspartokinase substrates. Since the inhibition of the dehydrogenase activity by aspartokinase substrates appears to be noncompetitive (Patte *et al.*, 1966), it is reasonable to assume that the aspartokinase substrates are binding to aspartokinase sites. Thus, by elimination, we conclude that the third possibility is the most reasonable explanation for the effects observed.

A more detailed description of the inactivation is not possible at present. In principle, it could occur either by an SH-TP-induced displacement of catalytic groups at the aspartokinase site(s) which prevents catalysis, or by the blockage of an essential conformational change or proton transfer during the catalytic process.

Desensitization of the Homoserine Dehydrogenase Activity to Threonine by Reaction with SH-TP. The desensitization of the dehydrogenase activity to threonine, after reaction of three sulfhydryl groups with SH-TP, cannot be explained by a dissociation of the enzyme, since the protein maintains its hexameric structure. Furthermore, in view of the evidence that only three SH-TP molecules react with the enzyme, whereas six sites exist for threonine binding (Janin *et al.*, 1969), it seems improbable in a protein of this size that the bound SH-TP molecules interfere with the binding of all of the threonine molecules by direct, steric interactions.

Three possible mechanisms could explain the desensitization of the homoserine dehydrogenase activity to threonine. One possibility, that reaction with SH-TP prevents threonine from binding by inducing a conformational change at the threonine sites, is in accord with the "induced-fit" concept of Koshland (1962). Alternatively, SH-TP could cause threonine desensitization by either blocking a threonine-induced conformational change at the dehydrogenase sites, or by "freezing" a preexisting allosteric equilibrium in a conformation which has a low affinity for threonine (Janin and Cohen, 1969). The last mechanism embodies certain ideas incorporated in the well-known model of Monod *et al.* (1965). Our evidence does not permit a choice to be made among these possibilities.

Reaction with SH-TP and the Subunit Structure of Aspartokinase I–Homoserine Dehydrogenase I. The results presented in this paper have shown that there are three unique sulfhydryl groups on aspartokinase I–homoserine dehydrogenase I which are associated with the aspartokinase activity. The simplicity of the kinetics of reaction with SH-TP suggests that these unique sulfhydryl groups have identical environments, and that they are independent of one another with respect to SH-TP reaction. Since, furthermore, the subunits of aspartokinase I–homoserine dehydrogenase I are identical, or nearly identical, in their peptide maps (Truffa-Bachi *et al.*, 1969), it seems reasonable to infer that the unique sulfhydryl groups are located on three identical subunits. Although other interpretations are not ruled out, they would require that only one or two of the subunits, instead of three, be unique in structure. Available evidence, particularly the evidence of only three homoserine dehydrogenase sites (Janin *et al.*, 1969; Heck and Truffa-Bachi, 1970), as well as the recent physical studies of Wampler *et al.* (1970) on the aspartokinase I–homoserine dehydrogenase I dimer, provides, however, no suggestion of one or two unique subunits, but strongly supports the possibility of three. Thus, three of the subunits in the hexamer seem to maintain the aspartokinase activity. The precise number of aspartokinase sites in this molecule remains, however, to be established.

Acknowledgment

We thank Dr. Joseph A. Duke and Miss Linda Stowring for the gift of SH-TP used in this work.

References

Black, S., and Wright, N. G. (1955), *J. Biol. Chem. 213*, 39.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Hampton, A., and Maguire, M. H. (1961), *J. Amer. Chem. Soc. 83*, 150.
Heck, H. d'A., and Truffa-Bachi, P. (1970), *Biochemistry 9*, 2776.
Janin, J., and Cohen, G. N. (1969), *Eur. J. Biochem. 11*, 520.
Janin, J., van Rapenbusch, R., Truffa-Bachi, P., and Cohen, G. N. (1969), *Eur. J. Biochem. 8*, 128.
Koshland, D. E. (1962), *in* Horizons in Biochemistry, Kasha, M., and Pullman, B., Ed., New York, N. Y., Academic Press, p 265.
Manders, W. W., Von Dippe, P. J., Horvath, M. L., Smith, V. A., and Smith, W. G. (1969), *J. Biol. Chem. 244*, 5510.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Murphy, A. J., Duke, J. A., and Stowring, L. (1970), *Arch. Biochem. Biophys. 137*, 297.
Murphy, A. J., and Morales, M. F. (1970), *Biochemistry 9*, 1528.
Patte, J.-C., Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 128*, 426.
Szentirmai, A., Szentirmai, M., and Umbarger, H. E. (1968), *J. Bacteriol. 95*, 1672.
Truffa-Bachi, P. (1968), Ph.D. Thesis, Faculté des Sciences, Paris, France.
Truffa-Bachi, P., Le Bras, G., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 128*, 440.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1968), *Eur. J. Biochem. 5*, 73.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1969), *Eur. J. Biochem. 7*, 401.
Wampler, D. E., Takahashi, M., and Westhead, E. W. (1970), *Biochemistry 9*, 4210.