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Enzyme Forms Produced from Aspartate Transcarbamoylase by Digestion with Trypsin

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1. The time-course of tryptic hydrolysis of aspartate transcarbamoylase (aspartate carbamoyltransferase, EC 2.1.3.2) was followed by activity measurements in the presence and absence of allosteric effectors, and by polyacrylamide-gel electrophoresis. 2. Two proteins with enzyme activity are formed in this way from native enzyme, and the isolation and some properties of these species are reported. The larger protein (10.6S) resembles native enzyme in that it contains regulatory subunits and is sensitive to allosteric effectors, as well as in a more detailed kinetic investigation. It appears from the time-course of tryptic digestion to be an intermediate in the formation of a catalytic subunit (5.5S) which is similar to, but not identical with, the catalytic subunit produced by mercurial treatment of the native enzyme. 3. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the different enzyme forms demonstrates that trypsin can hydrolyse bonds in the catalytic polypeptide chains as well as completely remove the regulatory polypeptide chains. 4. Both preparations of catalytic subunit can recombine with regulatory subunit to form enzymes which resemble the native enzyme in being activated by ATP, although they do not appear to be inhibited by CTP. 5. This study is consistent with the models of the enzyme that propose that the catalytic subunits are held together in the native enzyme by three pairs of regulatory polypeptide chains.

McClintock & Markus (1968) reported that tryptic hydrolysis of native aspartate transcarbamoylase resulted in complete loss of allosteric properties at an extent of digestion where no impairment of catalytic activity could be detected. It appeared that trypsin preferentially attacked the regulatory subunits of the enzyme. These and other studies (McClintock & Markus, 1968; Markus *et al.*, 1971) indicated that an investigation of the tryptic digestion of native aspartate transcarbamoylase would be of interest with regard to the structure of the enzyme. Two catalytically active proteins differing from the native enzyme have been isolated from limited tryptic digests of the native enzyme, and some properties of these species are reported.

Materials and Methods

Materials

Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; 2-mercaptoethanol and imidazole were from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; di-isopropyl phosphorofluoridate was from Boots, Nottingham, Notts., U.K., and sodium dodecyl sulphate was from Koch–Light, Colnbrook, Bucks., U.K. Sodium tetraborate, KCl and EDTA were the analytical reagent products of British Drug Houses Ltd., Poole, Dorset, U.K. Trypsin was the 2×crystallized product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. Dilithium carbamoyl phosphate (B grade), L-aspartic acid (A grade) and Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, A grade] were obtained from Calbiochem, La Jolla, Calif., U.S.A. ATP and CTP were purchased from P–L Biochemicals, Milwaukee, Wis., U.S.A. The strain of *Escherichia coli* (K12) was a gift from Dr. J. C. Gerhart, Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, U.S.A.

Enzyme preparation

Aspartate transcarbamoylase was prepared from *E. coli* (K12) by the procedures of Gerhart & Holoubek (1967) for the native enzyme, regulatory subunit and catalytic subunit (designated mercurial catalytic subunit).

Enzyme assay

The carbamoylaspartate produced at 28°C in 0.5ml reaction mixtures containing 0.05M-Tes buffer, adjusted to pH 8.0 with KOH, was determined colorimetrically as described by Gerhart & Pardee (1962).

Protein

This was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Tryptic hydrolysis of aspartate transcarbamoylase

Native enzyme (5.5mg/ml) was incubated at 28°C with trypsin (0.4mg/ml) in 0.05M-sodium borate buffer, pH8.5, containing 10mM-aspartate (McClintock & Markus, 1968), for various time-periods. The reaction was stopped by the addition of di-isopropyl phosphorofluoridate to give a final concentration of 0.1mM. The change in activity of the tryptic digestion mixture with time, measured in the presence and absence of ATP or CTP, is recorded in Fig. 1. Samples of the digestion mixture at times corresponding to those of the activity measurements in Fig. 1 were subjected to polyacrylamide-gel electrophoresis (Plate 1).

After digestion for 20min, the effects of 2mM-ATP and 0.2mM-CTP on the reaction are negligible, in agreement with the results of McClintock & Markus (1968), and the specific activity of the solution is higher than at zero time (Fig. 1). However, it is clear from Plate 1 that these observations cannot be interpreted simply, because a number of protein species are produced from the native enzyme. Band (A) corresponds in position to native enzyme and band (C) to the mercurial catalytic subunit. Band (C) appears to be formed via an intermediate, corresponding to band (B). After 90min of tryptic digestion only band (C) is present, and there is a marked fall in activity compared with the 60min digest. However, the amount of protein removed as acid-soluble peptides does not increase markedly in this time-period.

Preparation of catalytic subunit from a tryptic digest

A 20min digestion mixture as described above, containing 50mg of protein, was left for at least 1h at 0°C in 0.1mM-di-isopropyl phosphorofluoridate, and then concentrated tenfold. The sample was placed on top of a column (30cm × 3cm) of Sephadex G-100 in 0.04M-potassium phosphate buffer, pH7.0, containing 0.2mM-EDTA and 2mM-mercaptoethanol, and eluted with this buffer. Enzyme activity was associated with two protein peaks eluted just after the void volume of the column. Although the enzyme of the first peak was activated by 2mM-ATP and inhibited by 0.4mM-CTP, that of the second peak was inhibited by both nucleotides under these conditions although the inhibition by 0.4mM-CTP was barely significant. Samples of the proteins in both peaks were subjected to polyacrylamide-gel electrophoresis. The gel patterns showed that the second peak contained the protein corresponding to catalytic subunit, whereas the protein of the first peak appeared to be a

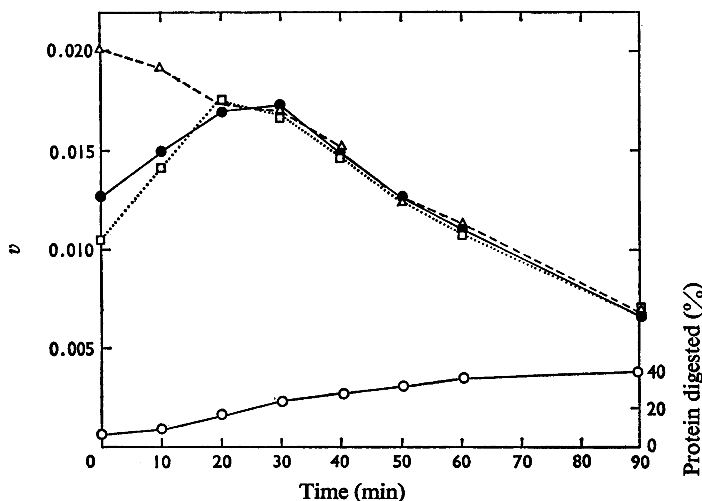


Fig. 1. *Time-course of tryptic digestion of aspartate transcarbamoylase*

The digestion mixture is described in the text. Assays were performed in 0.05M-Tes buffer, pH8.0, with 5mM-aspartate and 3.6mM-carbamoyl phosphate, (●) in the absence of nucleotides, (△) in the presence of 2mM-ATP and (□) in the presence of 0.2mM-CTP. Velocities are expressed as μmol of carbamoylaspartate formed/min per 0.04μl of digestion mixture. The percentage of protein digested (○) was determined by measuring the formation of $HClO_3$-soluble peptides, which were determined by the method of McClintock & Markus (1968).

Table 1. *Amino acid composition of the catalytic subunits and the intermediate enzyme form of aspartate transcarbamoylase*

Compositions in residues/molecule have been normalized to the leucine values of Weber (1968*a*), by using the revised molecular weights of the polypeptide chains (Weber, 1968*b*; Rosenbusch & Weber, 1971).

Amino acid	Catalytic subunit produced by ... Content [residues/molecule (mol.wt. 33000)]: *p*-Chloromercuribenzoate treatment		Tryptic hydrolysis	Content of native enzyme [residues/molecule (mol.wt. 300000)] (Weber, 1968*a*)	Content of intermediate protein†	Content of native enzyme less one regulatory dimer/molecule [residues/molecule (mol.wt. 266000)]	Content of intermediate protein*
	Weber (1968*a*)	Present work					
Lys	15.7	14.7	14.6	157.2	139.2	134.4	125.4
His	11.1	9.5	9.9	93.0	73.8	84.6	66.6
Arg	14.9	14.4	15.5	135.0	125.4	118.8	112.8
Asp	34.1	35.2	33.3	332.4	315.0	292.8	283.8
Thr	16.9	16.3	16.8	139.2	133.8	124.8	120.0
Ser	18.0	16.4	15.8	158.4	149.4	136.8	134.4
Glu	26.7	28.2	28.2	253.8	243.0	222.6	219.0
Pro	12.4	11.8	11.3	113.4	125.4	99.6	112.8
Gly	14.7	16.8	17.3	129.0	135.0	116.4	121.2
Ala	31.1	33.8	34.1	247.2	256.2	228.0	231.0
Val	21.5	21.5	21.3	201.6	184.2	177.0	166.2
Met	7.6	7.1	7.2	57.0	50.4	54.0	45.0
Ile	14.3	14.1	14.6	145.2	135.0	123.0	121.2
Leu	35.3	35.3	35.3	293.4	293.4	264.6	264.6
Tyr	8.5	6.1	6.1	63.6	53.4	58.2	52.8
Phe	11.4	11.2	11.7	97.2	96.6	87.6	87.0

* Calculations based on a leucine content of 264.6 residues, for comparison with native enzyme from which one of the three regulatory dimers has been removed.
† Calculations based on a leucine content of 293.4 residues, for comparison with native enzyme.

mixture of native enzyme and the intermediate corresponding to band (B) of Plate 1. The fractions comprising the second peak were pooled and concentrated, giving a yield of 10mg of protein. This preparation (designated tryptic catalytic subunit) appeared homogeneous during centrifugation in a Spinco model E ultracentrifuge at a rotor speed of 59000 rev./min for 1 h; analysis of the Schlieren patterns yielded a sedimentation coefficient of 5.5S at 6.5mg/ml and 20°C in the buffer used to elute it from Sephadex. This is similar to the value of 5.8S reported by Gerhart & Schachman (1965) for the mercurial catalytic subunit.

For a 1 mg/ml solution in 0.04M-potassium phosphate, pH7.0, containing 0.2mM-EDTA, with a light-path of 1 cm, $E_{280} = 0.92$ and $E_{280}/E_{260} = 1.32$. These values are similar to those observed on our preparation of the mercurial catalytic subunit, 0.83 and 1.36 respectively. The amino acid compositions of the catalytic subunits prepared by the two methods do not appear to differ significantly (Table 1).

Both preparations of catalytic subunit yield linear double-reciprocal plots of reaction velocity with respect to either substrate, and are inhibited by ATP as well as CTP. However, the tryptic catalytic subunit has a specific activity approximately one-quarter of that of the mercurial catalytic subunit, and although the two preparations appear to have the same reaction mechanism the kinetic constants determined for them are not identical.

A 90min tryptic digestion mixture was dialysed against 0.04M-potassium phosphate buffer, pH7.0, containing 0.2mM-EDTA and 0.2mM-mercaptoethanol. This preparation resembles the tryptic catalytic subunit in specific activity and in the kinetic constants which can be determined by the colorimetric assay for carbamoylaspartate.

Preparation of intermediates from tryptic digest

A 10min digestion mixture as described above, containing 108mg of protein, was left for at least 1 h at 0°C in 0.1 mM-di-isopropyl phosphorofluoridate. It was then placed on a column (70cm×1.5cm) of DEAE-Sephadex A-50 in 0.01M-imidazole–HCl buffer, pH7.0, and eluted with a 0.25–0.6M-KCl gradient (150ml of each). Fractions (3ml) were collected, and the elution pattern showed three absorption peaks at 280nm. Polyacrylamide-gel electrophoreses, performed and stained under the same conditions as for Plate 1, showed no protein component in the first peak, which nevertheless reacts with biuret reagent, native enzyme with a trace of intermediate in the second peak, and intermediate plus catalytic subunit in the third peak. Fractions 66 to 78, comprising the third peak, were pooled and concentrated to 2ml. This was then applied to a column (90cm× 2.5cm) of Sephadex G-200 and eluted with 0.04M-potassium phosphate buffer, pH7.0. There were two peaks of absorption at 280nm, the first containing the intermediate and the second the catalytic subunit plus a trace of intermediate. The fractions containing only intermediate were pooled and concentrated, giving a yield of 9mg of protein.

The preparation of the intermediate appeared homogeneous in the ultracentrifuge, with a sedimentation coefficient of 10.6S at 2mg/ml and 20°C in 0.04M-potassium phosphate buffer, pH7.0, containing 0.2mM-EDTA and 2mM-mercaptoethanol. This value is slightly lower than the value of 11.7S reported for the native enzyme after extrapolation to infinite dilution (Gerhart & Schachman, 1965).

For a 1 mg/ml solution with 1 cm light-path $E_{280} = 0.51$, which is similar to the value reported for native enzyme (Gerhart & Holoubek, 1967), and lower than the values for the catalytic subunits. The

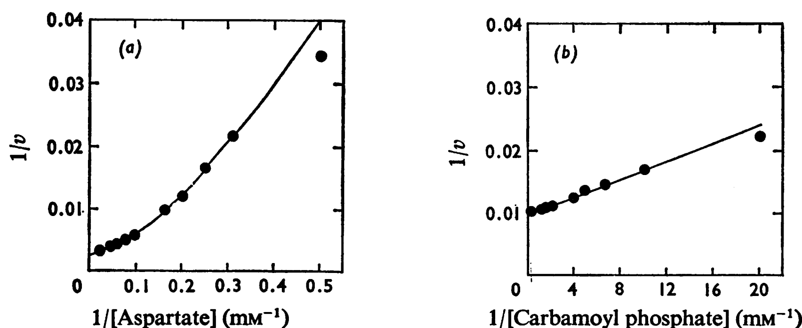
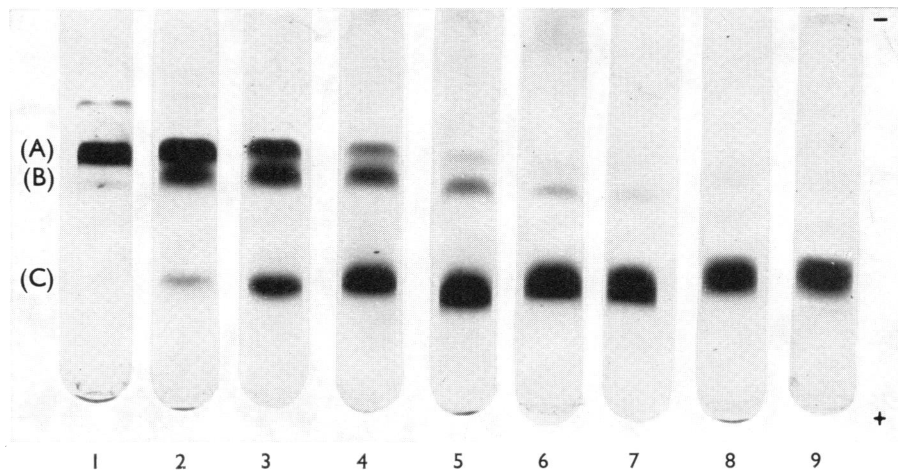


Fig. 2. *Effect of substrate concentration on the velocity of the reaction catalysed at pH8.0 by the intermediate form of the enzyme*

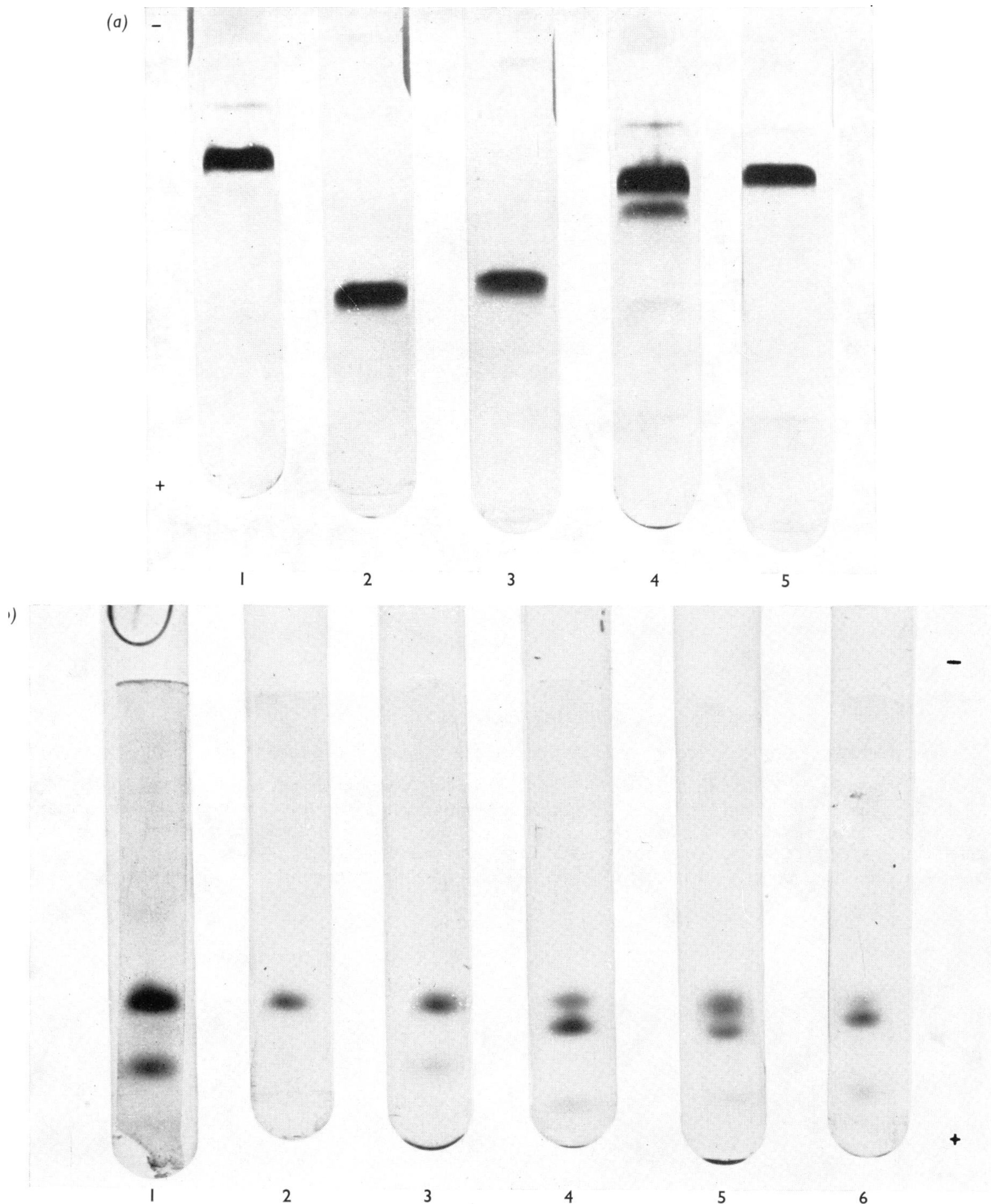
(*a*) Aspartate was varied and the carbamoyl phosphate concentration fixed at 1.0mM. (*b*) Carbamoyl phosphate was varied and the aspartate concentration fixed at 15mM. The amount of enzyme added per 0.5ml of assay mixture was 0.05 μg. Velocities are expressed as μmol of carbamoylaspartate formed/min per mg of enzyme.



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of aspartate transcarbamoylase after different periods of tryptic hydrolysis

The digestion mixture is described in the text. Electrophoreses were performed with a 6% resolving gel in triethanolamine–HCl buffer at pH 6.8 and 4°C. Direction of movement is from top to bottom. Protein was stained with Amido-Schwarz. Samples (1–8) from left to right correspond to the following periods of digestion at 28°C: 0 (1), 10 (2), 20 (3), 30 (4), 40 (5), 50 (6), 60 (7) and 90 (8) min. Sample (9) is catalytic subunit produced by mercurial treatment of native enzyme (Gerhart & Holoubek, 1967). For details of bands (A)–(C) see the text.



EXPLANATION OF PLATE 2

(a) Polyacrylamide-gel electrophoresis of the products of recombination between the catalytic subunits and the regulatory subunit and (b) sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of native aspartate transcarbamoylase and species produced from it

For 2(*a*) the conditions for recombination are described in the text. Samples from left to right are: (1) native enzyme; (2) mercurial catalytic subunit; (3) tryptic catalytic subunit; (4) enzyme reconstituted from tryptic catalytic subunit; (5) enzyme reconstituted from mercurial catalytic subunit. Electrophoreses were performed as described in the legend to Plate 1. For 2(*b*) the method used was that of Shapiro *et al.* (1967). Samples from left to right are: (1) native enzyme; (2) mercurial catalytic subunit; (3) intermediate form of enzyme; (4) tryptic catalytic subunit; (5) tryptic catalytic subunit plus mercurial catalytic subunit; (6) 90min digestion mixture. Migration is from top to bottom.

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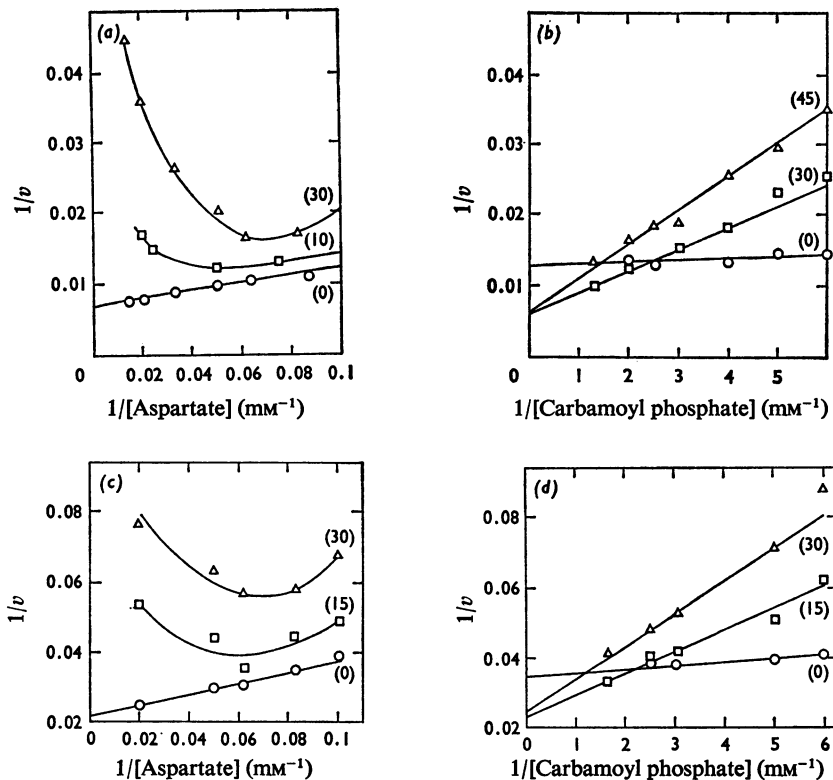

Fig. 3. *Product inhibition by phosphate of the reactions catalysed at pH 7.0 by the native and intermediate forms of aspartate transcarbamoylase*

(*a*) and (*b*), Native enzyme; (*c*) and (*d*), intermediate form of enzyme. (*a*) and (*c*), Aspartate was varied and the carbamoyl phosphate concentration fixed at 0.25mM. (*b*) and (*d*), Carbamoyl phosphate was varied and the aspartate concentration fixed at 10mM. The amount of enzyme added per 0.5ml of assay mixture was 0.05μg. Concentrations (mM) of phosphate added are shown in parentheses on the figure. Velocities are expressed as μmol of carbamoylaspartate formed/min per mg of enzyme.

amino acid composition of the intermediate is recorded in Table 1.

Kinetic properties of the native and intermediate forms of enzyme

For the intermediate, as for the native enzyme (Gerhart & Pardee, 1964), double-reciprocal plots of initial velocity with respect to aspartate concentration are non-linear, although similar plots with respect to carbamoyl phosphate concentration are apparently linear (Fig. 2). The pattern of product inhibition by phosphate is shown in Fig. 3. When phosphate is present, higher concentrations of aspartate appear to cause substrate inhibition of both enzyme forms (Figs. 3*a* and 3*c*). The patterns of the double-reciprocal plots when carbamoyl phosphate is the varied substrate are unusual in that phosphate appears to activate both forms of enzyme at the higher concentrations of carbamoyl phosphate although inhibition occurs at lower substrate concentrations (Figs. 3*b* and 3*d*). The latter patterns differ from the apparently competitive inhibition observed by Kleppe (1966). The specific activity of the intermediate, under the conditions of Fig. 3, is somewhat lower than that of the native enzyme.

Recombination of catalytic subunits with regulatory subunit

Samples of regulatory subunit, mercurial catalytic subunit and tryptic catalytic subunit were each dialysed against 0.04M-potassium phosphate buffer (pH 7.0) for 24h and the protein concentration of each sample was determined. Regulatory subunit (270μg) was then added to 100μg samples of each catalytic

Table 2. *Effect of ATP and CTP on the activity of enzyme reconstituted from catalytic and regulatory subunits*

The assay mixture contained 0.05M-Tes (pH 8.0), 1mM-carbamoyl phosphate, 5mM-aspartate and nucleotide, when present, at 2mM.

Enzyme	Percentage activation (+) or inhibition (−) in presence of	
	ATP	CTP
Native enzyme	+48	−30
Intermediate	+41*	−19†
Mercurial catalytic subunit	−24	−17
Tryptic catalytic subunit	−11	−6
Recombined:		
Mercurial catalytic+regulatory subunits	+24	—
Tryptic catalytic+regulatory subunits	+23	+8

* Value obtained at an aspartate concentration of 4mM.
† Value obtained at a carbamoyl phosphate concentration of 0.25mM.

subunit, in total volumes of 0.3ml, to give mixtures where the molar ratio of catalytic subunit to regulatory subunit was approx. 1:8. The mixtures were dialysed for 24h against 0.04M-potassium phosphate buffer (pH 7.0) containing 0.1mM-mercaptoethanol (Gerhart & Schachman, 1965).

The recombination products were subjected to polyacrylamide-gel electrophoresis (Plate 2*a*). It appears on this basis that the product from the mercurial catalytic subunit is native enzyme, whereas the tryptic catalytic subunit gives rise to some of the intermediate enzyme form as well as native enzyme.

The results of assaying the recombination products in the presence and absence of nucleotides are recorded in Table 2, together with appropriate controls by using the native enzyme and the catalytic subunits. The reconstituted enzymes are activated by ATP, although to a smaller extent than the untreated native enzyme. However, they do not appear to be inhibited by CTP, in contrast with the report of Gerhart & Schachman (1965). Indeed, the enzyme reconstituted from tryptic catalytic subunit appears to be significantly activated by CTP (Table 2). The specific activities of the reconstituted proteins were approximately half of those of the corresponding catalytic subunits. The enzyme reconstituted from mercurial catalytic subunit therefore resembles untreated native enzyme in specific activity, whereas enzyme reconstituted from tryptic catalytic subunit has a lower activity. Thus the two reconstituted enzymes differ from untreated native enzyme in their reaction to CTP and possibly also in specific activity, although the three proteins appear identical in Plate 2*a*.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Samples of native enzyme and the species produced from it have been subjected to sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by the method of Shapiro *et al.* (1967). The native enzmye and the intermediate have a fast-moving component in the same position (Plate 2*b*) and on this basis it is concluded that the intermediate contains regulatory subunit as well as catalytic subunit. The tryptic catalytic subunit shows three bands; the top band corresponds in position to single catalytic polypeptides of the type produced from mercurial catalytic subunit, as shown by the higher relative intensity of the top band in the gel on which both mercurial and tryptic catalytic subunits were placed (Plate 2*b*). Hence the middle band for the tryptic catalytic subunit probably corresponds to a protein of molecular weight somewhat less than 33000 (Weber, 1968*b*; Rosenbusch & Weber, 1971), and the lowest band to a molecular weight lower than that of the regulatory subunit.

Although the tryptic catalytic subunit appears to contain three different protein molecules (Plate 2*b*) it was not possible to demonstrate any extra *N*-terminal amino acids, compared with the mercurial catalytic subunit, by using the standard procedure for proteins of Gros & Labouesse (1969).

A 90min tryptic digest has approximately one-third of the specific activity of a 20min digest, and appears in Plate 1 to be identical with catalytic subunit. In Plate 2*b* the 90min digest shows a pattern similar to that of the tryptic catalytic subunit, although the intensity of the top band may be weaker relative to the middle band, indicating a lower proportion of polypeptides of molecular weight 33000.

Digestion of mercurial catalytic subunit with trypsin

Mercurial catalytic subunit was incubated with trypsin under conditions similar to those used with the native enzyme to give Fig. 1. Samples were treated with di-isopropyl phosphorofluoridate after 15 and

30min, and subjected to sodium dodecyl sulphate electrophoresis. A pattern of three bands was obtained, similar to that shown in Plate 2*b* for the tryptic catalytic subunit.

Discussion

McClintock & Markus (1968) reported that tryptic hydrolysis of the enzyme resulted in a complete loss of allosteric properties at an extent of digestion at which no impairment of catalytic activity could be observed. The present results show an increase in activity of the digestion mixture during the first 20min, which is possibly a result of a higher specific activity of freshly separated catalytic subunit relative to that of the native enzyme (Gerhart & Holoubek, 1967). At the stage where catalytic activity of the digestion mixture is at a maximum, the reason for the lack of an effect of ATP or CTP on the reaction velocity is complex. Although the native enzyme and the mixture of species in the first peak from the Sephadex G-100 column are activated by ATP and inhibited by CTP, the catalytic subunit in the second peak is inhibited by 2mM-ATP and also to a very small extent by 0.4mM-CTP. Thus the ATP effects cancel out, although the CTP effect simply appears insignificant at that concentration. However, higher concentrations of CTP clearly inhibit the catalytic subunit.

The results of polyacrylamide-gel electrophoresis (Plate 1) are consistent with the tryptic removal of regulatory subunits in a process which is not one-stage, because of the appearance of the intermediate protein, which still contains regulatory subunits (Plate 2*b*). On incubation over longer time-periods, all the regulatory subunit is removed and trypsin apparently attacks the catalytic subunits. Thus sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the tryptic catalytic subunit (Plate 2*b*) indicates that some of the catalytic polypeptides have been somewhat diminished in size, and in addition a much smaller polypeptide can be seen. It is noteworthy that the tryptic catalytic subunit gives rise to three protein bands on electrophoresis in the presence of sodium dodecyl sulphate (Plate 2*b*), but only one rather wide band in its absence (Plates 1 and 2*a*). Thus some if not all of the peptides produced from the catalytic polypeptides by tryptic digestion appear to remain attached by non-covalent bonds to the rest of the catalytic subunit. This conclusion is supported by the similarity of the sedimentation coefficients and amino acid compositions of the tryptic and mercurial catalytic subunits.

The specific activity of the tryptic catalytic subunit is approximately one-quarter of that of the mercurial catalytic subunit. From the appearance in the tryptic catalytic subunit of modified polypeptides close to the position of the original catalytic type (Plate 2*b*), it seems that some of the catalytic polypeptides have been partly digested. These results suggest that the partly digested polypeptides could be inactive, thus accounting for the lower specific activity. There is no kinetic evidence for the presence of two different types of active site, since under all conditions used in a detailed kinetic study of the reaction mechanism double-reciprocal plots of kinetic results were linear (E. Heyde, A. Nagabhushanam & J. F. Morrison, unpublished work). However, some of the kinetic constants determined for the tryptic catalytic subunit are significantly different from those for the mercurial catalytic subunit (E. Heyde, A. Nagabhushanam, & J. F. Morrison, unpublished work), as also are the degrees of inhibition by nucleotides shown in Table 2. Therefore it is possible that tryptic splitting of one polypeptide in the catalytic subunit leads to inactivation at that site and to a change in the catalytic properties of the neighbouring intact polypeptides. Against this interpretation it should be noted that the specific activity and kinetic properties of a 90min digestion mixture are similar to those of the isolated tryptic catalytic subunit, even though the former preparation seems to contain a greater proportion of partly digested polypeptides (Plate 2*b*). Therefore it remains a possibility that the partly digested polypeptides are not inactive and that a conformational change affecting all the active sites in the catalytic subunit occurs as a consequence of tryptic hydrolysis in any or all of the polypeptides.

The tryptic catalytic subunit combines with regulatory subunit to form reconstituted native enzyme and apparently a minor amount of the intermediate protein (Plate 2*a*). This observation, together with evidence that the latter contains regulatory subunit (Plate 2*b*), indicated that the essential difference between the native and intermediate proteins might be in the catalytic subunits. That this is not so is shown by the appearance of only one band corresponding to catalytic polypeptide on sodium dodecyl sulphate electrophoresis of the intermediate (Plate 2*b*). The kinetic properties of the native and intermediate forms of enzyme are very similar, as are their amino acid compositions. However, from the gel-electrophoresis patterns and the measurements of sedimentation coefficients it appears that the intermediate form is slightly smaller than the native enzyme, and this is supported by a comparison of the amino acid compositions given in Table 1. Thus, when the compositions are normalized on the basis of the leucine contents, the intermediate is seen to contain less of the majority of the amino acids than does the native enzyme. The second set of values for the intermediate (Table 1) has been expressed so as to test the hypothesis that one of the three regulatory dimers may have been removed from the native enzyme during the formation of the intermediate. Whereas the composition of the intermediate is consistent with this hypothesis for the majority of the amino acids, the

contents of the least abundant amino acids, histidine, proline, methionine and tyrosine, differ by −21% to +13% from the expected values. Hence it is still possible that tryptic digestion has not been limited to a single regulatory dimer up to the stage at which the intermediate is formed. However, it seems clear that the difference between native enzyme and the intermediate shown in Plate 1 is the result of changes in size as well as conformation and/or charge, brought about by limited digestion of the regulatory subunits. In this connexion it is noteworthy that the intermediate form is less sensitive than the native enzyme to inhibition by CTP and to activation by ATP (Table 2), which also indicates that a change has occurred affecting the regulatory subunits.

Enzyme reconstituted by mixing separate preparations of catalytic subunit and regulatory subunit resembled native enzyme in being activated by ATP, but differed in being unaffected by CTP at a concentration that inhibits the native enzyme (Table 2). In the latter respect the present results differ from the report by Rosenbusch & Weber (1971) that enzyme reconstituted under different conditions showed CTP inhibition similar to that observed with native enzyme. On the other hand, Gerhart (1970) reported that CTP inhibition of reconstituted enzyme was not as effective as that of the native enzyme. It is probable that on storage the regulatory subunit undergoes changes which affect not only the efficiency of recombination but also the sensitivity of the reconstituted enzyme to CTP. Zn^{2+} may be involved in both aspects (Rosenbusch & Weber, 1971; see Fig. 9 of Gerhart, 1970). It may well be that variation in the degree of such changes in the preparations of regulatory subunit used for recombination experiments has led to different properties in the recombination products. The regulatory subunit used in the present work had been separated from the catalytic subunit for approximately 1 month.

The present study of the tryptic digestion of aspartate transcarbamoylase is consistent with the models of the enzyme illustrated by Markus *et al.* (1971), Rosenbusch & Weber (1971) and Gerhart (1970). In all cases it is proposed that the two catalytic subunits, each containing three polypeptide chains, are held together by three pairs of regulatory polypeptide chains. Tryptic digestion appears to occur via an initial stage in which only the regulatory subunits are affected, followed by complete removal of the regulatory subunits and a simultaneous limited digestion of the catalytic polypeptides. It is also possible to interpret the present results in terms of a model where the two catalytic subunits are in juxtaposition, with the regulatory subunits on the outside of the catalytic subunits. However, this structure is considered less likely because of the failure to observe a large catalytic subunit, equivalent to six catalytic polypeptide chains, either during the course of tryptic digestion or on treatment with *p*-chloromercuribenzoate. If the regulatory subunits are indeed on the outside of the molecule of native enzyme, their presence must be essential for the integrity of the central structure involving catalytic polypeptides.

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