BBA 66514

STUDIES ON THE RELATIONSHIP BETWEEN THE ACTIVE SITES OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE FROM *ESCHERICHIA COLI* OR *AEROBACTER AEROGENES*

G. L. E. KOCH*, D. C. SHAW AND F. GIBSON

Biochemistry Department, John Curtin School of Medical Research, Institute of Advanced Studies, Australian National University, Canberra, A.C.T. (Australia)

(Received August 24th, 1971)

SUMMARY

1. During the overall conversion of chorismate (and NAD^+) to 4-hydroxyphenylpyruvate (and NADH) by chorismate mutase–prephenate dehydrogenase from *Escherichia coli*, the small amount of prephenate found to accumulate in the reaction mixture could not account for the observed rate of the dehydrogenase reaction.

2. The overall reaction catalysed by the enzyme from *Aerobacter aerogenes*, measured by NADH formation with chorismate and NAD^+ as substrates proceeded at a linear rate without lag.

3. The chorismate mutase reaction catalysed by the enzymes from *E. coli* or *A. aerogenes* was inhibited by prephenate. The enzyme from *A. aerogenes* was inhibited competitively by prephenate and the K_i for prephenate was very similar to its K_m in the dehydrogenase reaction.

4. Various protein-modifying reagents, including iodoacetamide, were found to inactivate the enzyme from *E. coli* and there was parallel inactivation of the chorismate mutase and prephenate dehydrogenase activities. Chorismate or prephenate protected both chorismate mutase and prephenate dehydrogenase activities to the same extent.

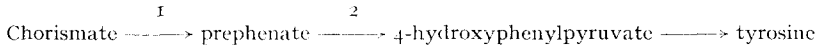
5. During the reaction of the enzyme with iodoacetamide, a single cysteinyl residue was protected by prephenate. Thus modification of only one residue results in the inactivation of both enzymic functions.

6. It was concluded that the available information favours a model for the enzyme in which the mutase and dehydrogenase sites are close to, or contiguous with, one another and that prephenate is probably a bound intermediate.

INTRODUCTION

The enzyme chorismate mutase–prephenate dehydrogenase catalyses two of

* Present address: M.R.C. Laboratory of Molecular Biology, University Postgraduate Medical School, Hills Road, Cambridge CB2 2QH, England.



Scheme 1. The terminal reactions in the biosynthesis of tyrosine in the enteric bacteria. The relevant enzymic activities are: 1, chorismate mutase; 2, prephenate dehydrogenase (requires NAD^+).

the terminal reactions in tyrosine biosynthesis in the enteric bacteria (Scheme 1) and previous studies have shown the enzyme to contain identical or, at least, very similar subunits[1,2]. Such enzymes having only one type of polypeptide chain but possessing more than one catalytic function, have been named "multifunctional" proteins[3]. The recent discovery of a substantial number of these proteins has increased interest in the mechanism by which they have evolved and the selective advantages they might confer on an organism. Evidence for a general mechanism by which more than one catalytic function could have evolved onto a single polypeptide chain has been obtained by YOURNO *et al.*[4]. It was shown that a single composite polypeptide made up of two previously independent enzymes could be produced, apparently as a result of deletion of the region between the cistrons coding for the two enzymes. Thus the feasibility of a model for the evolution of multifunctional proteins through the fusion of separate enzymes has been demonstrated.

However, the nature of the selective advantage conferred on an organism by the presence of multifunctional proteins remains uncertain. A potential advantage would be the capacity to channel intermediates in sequential reactions, thus possibly increasing the catalytic efficiency of the overall process and permitting co-ordinated regulation of the metabolic pathway concerned. Histidinol dehydrogenase appears to exemplify this possibility since the intermediate, histidinal, remains enzyme-bound during the conversion of histidinol to histidine[5]. However the same is not true of several other bifunctional proteins. In the case of aspartokinase-homoserine dehydrogenase, such channelling is not possible as the reactions are not sequential[6]. Indoleglycerolphosphate synthetase[7] and chorismate mutase–prephenate dehydratase[8] appear to possess mechanisms which involve 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate and prephenate respectively as free intermediates during the overall reactions they catalyse. This suggests that an increase in catalytic efficiency of the overall reaction may not necessarily result from the association of two sequential catalytic functions into a single complex.

The present paper reports experiments designed to elucidate the relationship between the mutase and dehydrogenase functions of chorismate mutase–prephenate dehydrogenase.

MATERIALS AND METHODS

Reagents and enzymes

Chorismic acid was prepared and purified by the method of GIBSON[9]. Prephenate was prepared as described previously[10]. Iodoacetamide (Sigma Chemical Co., Mo., U.S.A.) was purified by recrystallisation from ethyl acetate–light petroleum. [^{14}C]Iodoacetamide (52.3 mC/mmole) was purchased from the Amersham Radiochemical Centre, U.K. Carboxypeptidase A was treated with diisopropylphosphoryl fluoridate before use[11]. Chorismate mutase–prephenate dehydrogenase from *Aerobacter aerogenes* and *Escherichia coli* were purified by the procedures described previous-

ly[10,12]. All other reagents were obtained commercially and not further purified.

Assays for the chorismate mutase and prephenate dehydrogenase activity

The 20 min assays for chorismate mutase and prephenate dehydrogenase, and the procedure for the determination of initial rates of the two reactions have been described previously[10,12].

Assay of the overall reaction from chorismate to 4-hydroxyphenylpyruvate

The rates of the chorismate mutase and prephenate dehydrogenase functions during the overall reaction which converts chorismate and NAD^+ to 4-hydroxyphenylpyruvate and NADH were determined by continuous recording in a Cary 14 spectrophotometer. Since it was not possible to monitor the 2 reactions simultaneously, duplicate samples containing the required reaction mixtures were prepared. The rate of the chorismate mutase reaction was determined by measuring the decrease in absorbance at 274 nm using a molar extinction coefficient of 2630 for chorismate[13]. The rate of the dehydrogenase reaction was determined by measuring the rate of increase in absorbance at 340 nm using the second sample under conditions identical with those used for the mutase reaction. A value of 6200 was used for the molar extinction coefficient of NADH[14]. The high absorbance of NAD^+ at 274 nm precluded its use at concentrations above $2 \cdot 10^{-4}$ M in the assay for chorismate mutase. Therefore in the experiments in which the overall reaction required an NAD^+ concentration of $5 \cdot 10^{-4}$ M, the mutase reaction was measured separately in the absence of NAD^+. A correction of 10% was then made to the rate to account for the stimulation by NAD^+ of the mutase reaction catalysed by the enzyme from *E. coli*. This correction was determined from a study of the effect of NAD^+ concentration on the mutase reaction (unpublished results).

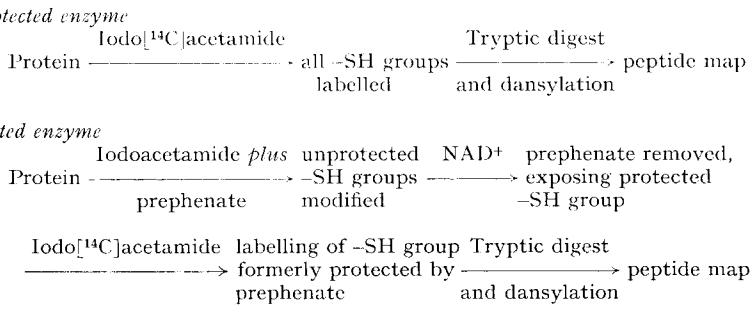
The reactions were carried out at 37° in 0.1 M Tris–HCl buffer (pH 8.1) containing 10^{-3} M EDTA and were started by the addition of enzyme. A period of 20 sec was normally allowed for mixing and stabilising before commencing recording of the reaction.

Modification of the enzyme with protein modifiers

Samples of the enzyme (10 μM) were prepared in 0.1 M Tris–HCl buffer (pH 7.4) containing 10^{-3} M EDTA. The modifying reagents were usually added at 100 times the molar concentration of the enzyme (*i.e.* 10^{-3} M). Carboxypeptidase A when used, was included at a final concentration of 20 μg/ml. The mixture of enzyme and modifier was incubated at 37° for 30 min after which time aliquots were removed and assayed for chorismate mutase and prephenate dehydrogenase activity. The dilution for the assays (about 1000-fold) was sufficient to eliminate any further significant inhibitory effects of the protein modifier on the enzymic reactions. Any variations in the concentration of the modifiers or the period of incubation are indicated in RESULTS. For studies on the protection of the enzyme, the conditions used were as described above with the potential protecting agents being added to the reaction mixture at a final concentration of 10^{-3} M.

Labelling of the enzyme with radioactive iodoacetamide

The procedure used to label the active site(s) of the enzyme with iodo[^{14}C]acet-



Scheme 2. Outline of procedures used to label the enzyme with radioactive iodoacetamide and to identify the tryptic peptide containing the amino acid residue protected by prephenate against reaction with iodoacetamide.

amide is illustrated in Scheme 2. One aliquot of the enzyme ("unprotected enzyme") was treated with iodo[^{14}C]acetamide of known specific activity under the conditions described above for protein modifiers. A second aliquot ("protected enzyme") was treated with non-radioactive iodoacetamide in the presence of 10^{-3} M prephenate and incubated for 60 min at 37°. At the end of this period the reaction mixture was made 10^{-2} M with respect to NAD^+ and radioactive iodoacetamide added to make the specific radioactivity equal to that in the first sample. The incubation was continued until the residual enzyme activities were negligible. Dithiothreitol was added to remove excess iodoacetamide and the labelled proteins dialysed and lyophilised. The samples were then oxidised with performic acid and maps of the dansylated tryptic peptides prepared as described previously. Sheets of Kodak Blue Brand Medical X-Ray film were exposed to the developed chromatograms in darkness for at least 5 days. The radioactivity in spots was quantitated by removing them from the plate, mixing with 5 ml of scintillation fluid in a vial, and counting in a Packard Scintillation Counter.

RESULTS

Comparison of the rates of the chorismate mutase and prephenate dehydrogenase reactions during the overall reaction

The procedure described in MATERIALS AND METHODS was used to determine the rates of the partial reactions during the overall conversion of chorismate into 4-hydroxyphenylpyruvate by the enzyme from *E. coli*. The NAD^+ concentration was maintained at saturating levels to avoid retardation of the dehydrogenase step. The rate of the chorismate mutase reaction was linear throughout the period during which chorismate utilisation was monitored. In the experiment shown (Fig. 1) the rate was 0.21 μmole chorismate utilised per min. The prephenate dehydrogenase reaction, however, commenced at a rate of about 0.13 μmole NADH formed per min and rapidly increased to a linear rate of 0.21 μmole NADH formed per min. The lag, namely that period elapsing between the start of the reaction and the attainment of the linear rate, was about 25 sec. The amount of prephenate accumulating in the reaction mixture during the overall reaction was calculated from the difference in the amount of chorismate utilised and NADH formed. Fig. 1 shows that the maximum

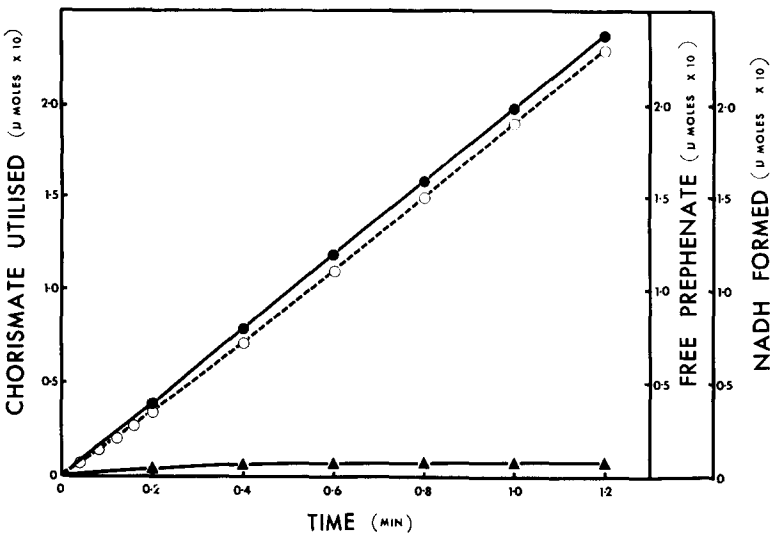


Fig. 1. Comparison of the rates of chorismate utilization, NADH formation and prephenate formation, during the overall reaction catalysed by chorismate mutase–prephenate dehydrogenase from *E. coli*. The procedure used is described in MATERIALS AND METHODS. The chorismate concentration was $4 \cdot 10^{-4}$ M and the NAD^+ concentration was $5 \cdot 10^{-4}$ M. ●—●, chorismate utilized; ○---○, NADH formed; ▲—▲, free prephenate. The rate of reaction in this and other figures was monitored by continuous recording.

concentration of free prephenate at any time was $7 \cdot 10^{-6}$ M. When the rate of the dehydrogenase reaction was determined using the conditions described for the overall reaction in Fig. 1, and replacing chorismate with $7 \cdot 10^{-6}$ M prephenate, the rate of NADH formation was negligible compared with that observed during the overall reaction from chorismate (Fig. 2).

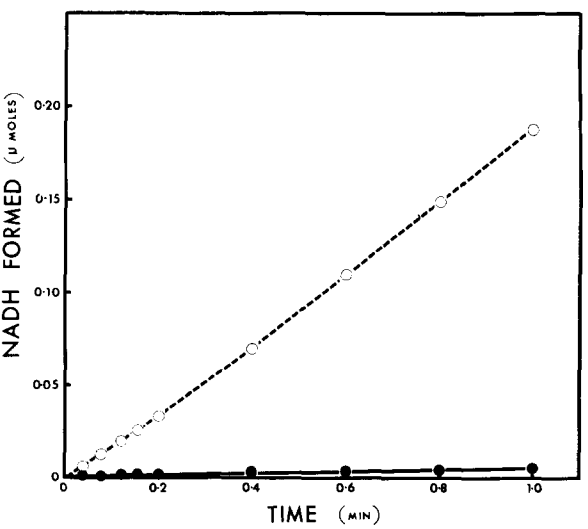


Fig. 2. Comparison of the rate of formation of NADH obtained during the overall reaction form chorismate with that obtained using prephenate. ○---○, overall reaction using the conditions described in Fig. 1; ●—●, reaction with $7 \cdot 10^{-6}$ M prephenate and $5 \cdot 10^{-4}$ M NAD^+.

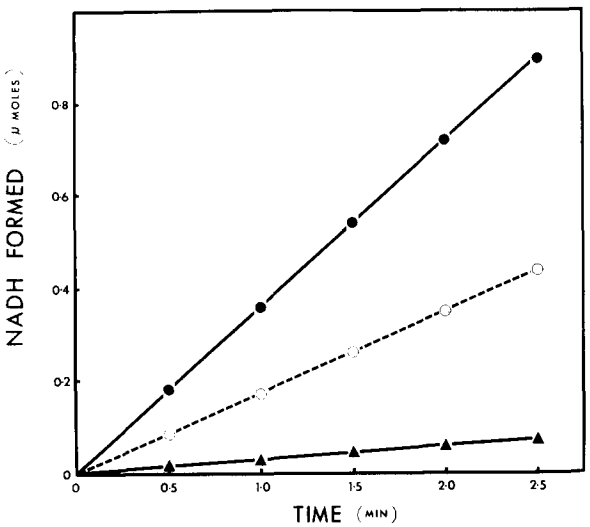


Fig. 3. The rate of formation of NADH during the overall reaction catalysed by chorismate mutase–prephenate dehydrogenase from *A. aerogenes*. The enzyme was incubated with $5 \cdot 10^{-4}$ M chorismate and 10^{-3} M NAD^+, the rate of formation of NADH was monitored by continuous recording. ●—●, 10 μl enzyme solution; ○---○, 5 μl enzyme solution; ▲—▲, 1 μl enzyme solution.

The specific activities of the chorismate mutase and prephenate dehydrogenase reactions were also determined separately with the enzyme from *E. coli* using the conditions described for the overall reaction. Chorismate and prephenate were each used at concentrations of $4 \cdot 10^{-4}$ M and NAD^+ at $5 \cdot 10^{-4}$ M. The same enzyme concentration was used for both assays. The chorismate mutase activity was 0.33 μmole chorismate utilised per min and the prephenate dehydrogenase activity was 0.51 μmole NADH formed per min. Therefore the turnover number for prephenate dehydrogenase was about 60% higher than that for chorismate mutase under the conditions used.

Unlike the reaction catalysed by the enzyme from *E. coli* (see above) no lag could be detected in the rate of formation of NADH upon incubation of the enzyme from *A. aerogenes* with chorismate and NAD^+. NADH formation proceeded linearly throughout the assay period (Fig. 3) even when the enzyme concentration was decreased to the lowest possible level permitting detection of activity or when the period prior to the start of the recording was reduced to 10 sec.

The effect of chorismate on prephenate dehydrogenase

The possibility that chorismate was an activator of prephenate dehydrogenase was checked by the addition of $5 \cdot 10^{-4}$ M chorismate to the assay mixture containing $5 \cdot 10^{-4}$ M prephenate and $5 \cdot 10^{-4}$ M NAD^+ using the enzyme from *E. coli*. The rate of prephenate dehydrogenase activity only increased about 3% in the presence of chorismate.

The effect of prephenate on chorismate mutase

The addition of prephenate to the assay for chorismate mutase from both *A. aerogenes* and *E. coli* caused a marked inhibition of both enzymes. The enzyme

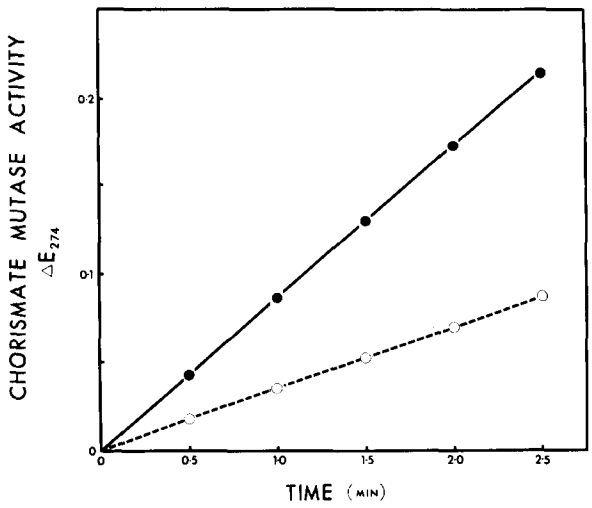


Fig. 4. Inhibition of chorismate mutase activity by prephenate, using the enzyme from *E. coli*. ●—●, without prephenate; ○---○, with 10^{-3} M prephenate.

from *E. coli* was inhibited 60% (Fig. 4) and that from *A. aerogenes* 54% by 10^{-3} M prephenate. The chorismate mutase activity of the enzyme from *E. coli* gave non-linear double reciprocal plots and the kinetics of prephenate inhibition were not studied further. Double reciprocal plots obtained with and without prephenate using the enzyme from *A. aerogenes* were clearly linear and intersected on the abscissa (Fig. 5) indicating that prephenate was a competitive inhibitor of chorismate mutase. The K_i for prephenate, $3.1 \cdot 10^{-4}$ M, is similar to the K_m for prephenate in the dehydrogenase reaction, which under the same conditions was found to be $3.5 \cdot 10^{-4}$ M.

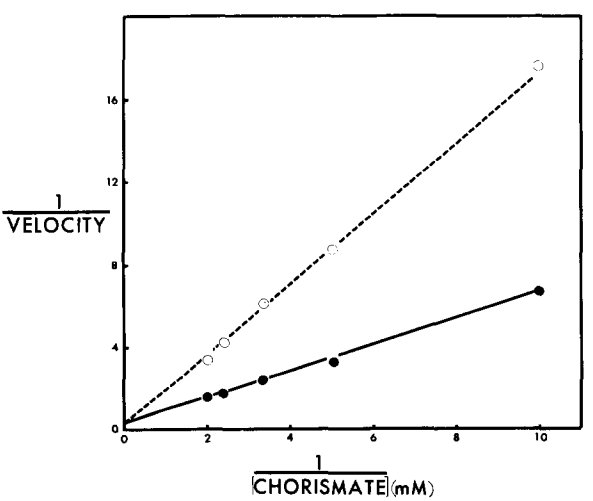


Fig. 5. Estimation of K_i for prephenate in the chorismate mutase reaction catalysed by the enzyme from *A. aerogenes*. ●—●, without prephenate; ○---○, with $5 \cdot 10^{-4}$ M prephenate.

The effect of protein-modifying reagents on the chorismate mutase and prephenate dehydrogenase activities

The enzyme from *E. coli* was treated with a variety of reagents known to modify amino acid residues in proteins. The procedure used is described in MATERIALS AND METHODS. With every modifier tested the inactivation of the mutase and dehydrogenase functions occurred concurrently and no significant differences could be detected between the two functions in either the rates at which they were inactivated (Table I) or in their dependence on the concentration of the various modifying agents (Table II).

TABLE I

THE RATES OF INACTIVATION OF THE ENZYMIC FUNCTIONS OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE FROM *E. coli* BY PROTEIN-MODIFYING REAGENTS

Conditions were as described in MATERIALS AND METHODS and samples were assayed for activity at the times shown.

Modifying reagent	*Enzymic function*	*Residual activity (%) at*			
		5 min	*10 min*	*20 min*	*30 min*
Tetranitromethane	Mutase	7	0	0	0
	Dehydrogenase	5	0	0	0
N-Bromosuccinimide	Mutase	25	4	0	0
	Dehydrogenase	29	7	0	0
Iodoacetic acid	Mutase	80	63	41	30
	Dehydrogenase	85	63	38	28
Iodoacetamide	Mutase	73	56	33	22
	Dehydrogenase	78	54	30	20
N-Ethylmaleimide	Mutase	66	32	18	10
	Dehydrogenase	61	32	23	10
Carboxypeptidase A	Mutase	65	47	22	10
	Dehydrogenase	65	42	26	13

The effects of prephenate, chorismate, NAD^+ and tyrosine on the action of modifying reagents

The four most likely protecting reagents for the enzyme were tested for their ability to prevent inactivation by the modifying reagents used above. The results show that only chorismate and prephenate have protective effects on the enzyme (Table III). It is also clear that in all cases the mutase and dehydrogenase functions are affected similarly and differential protection is not observed with either chorismate or prephenate.

Labelling and identification of the residue(s) reacting with iodoacetamide and protected by prephenate

The procedures used for the preparation of tryptic peptide maps of "protected" and "unprotected" enzyme are described in MATERIALS AND METHODS and outlined in Scheme 2. In the present context the term "protected enzyme" refers to that species selectively labelled with iodo[^{14}C]acetamide after previous exposure to unlabelled iodoacetamide in the presence of prephenate. "Unprotected enzyme" refers to that labelled directly with iodo[^{14}C]acetamide in the absence of prephenate. Radioautographs of dansyl tryptic peptide maps of the unprotected enzyme revealed

TABLE II

THE EFFECTS OF VARIED CONCENTRATIONS OF MODIFYING REAGENT ON THE ENZYMIC FUNCTIONS OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE FROM *E. coli*

The conditions used were as described in MATERIALS AND METHODS, except that the reagents were used at the concentrations shown.

Modifying reagent	*Concn. (mM)*	*Residual activity (%)*	
		Mutase	*Dehydrogenase*
Tetranitromethane	1	0	0
	0.1	0	0
	0.01	20	14
N-Bromosuccinimide	1	0	0
	0.1	12	9
	0.01	41	48
Iodoacetamide	1	22	15
	0.1	68	66
	0.01	95	100
N-Ethylmaleimide	1	10	10
	0.1	26	23
	0.01	94	100

the presence of two strongly labelled peptides near the origin in addition to four weakly labelled ones (Fig. 6a). Scintillation counting of each spot after elution showed that the strongly labelled peptides contained about 4 times as much radioactivity as any of the weaker ones. The radioactive material remaining at the origin does not

TABLE III

THE EFFECTS OF POTENTIAL PROTECTING REAGENTS ON THE INACTIVATION OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE FROM *E. coli* BY PROTEIN-MODIFYING REAGENTS

*Protein-modifying reagent**	*Potential protecting reagent***	*Residual activity (%)*	
		Mutase	*Dehydrogenase*
Tetranitromethane	None	0	0
	Prephenate	0	0
	Chorismate	0	0
	NAD^+	0	0
Iodoacetamide	None	22	20
	Prephenate	85	92
	Chorismate	80	88
	Tyrosine	20	20
	NAD^+	24	20
N-Ethylmaleimide	None	10	10
	Prephenate	70	62
	Chorismate	65	60
	Tyrosine	12	16
	NAD^+	10	16
Carboxypeptidase A	None	10	13
	Prephenate	70	70
	Chorismate	85	85
	Tyrosine	15	12
	NAD^+	11	8

* The conditions used were the same as those in Table I, the samples were assayed after 30 min.

** The potential protecting reagents were used at a concentration of 10^{-3} M.

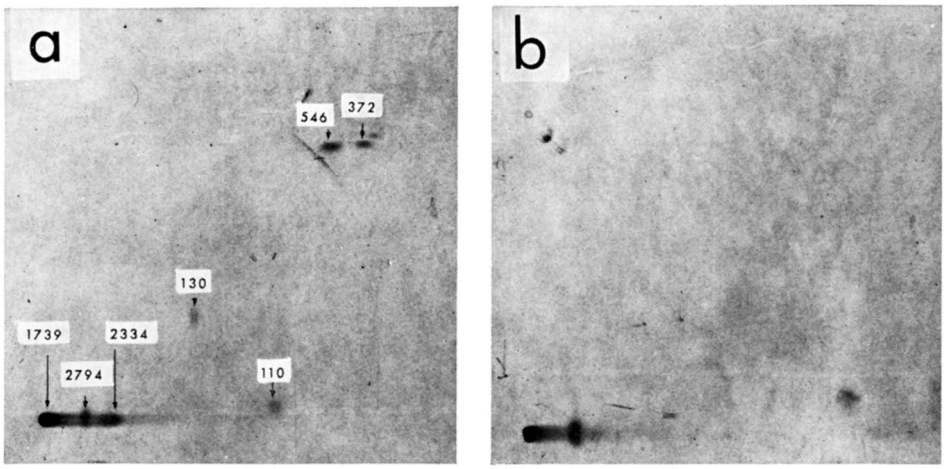


Fig. 6. Radioautographs of dansyl peptide maps of (a) 'unprotected' and (b) 'protected' chorismate mutase–prephenate dehydrogenase from *E. coli* after treatment with radioactive iodoacetamide by the procedure described in Scheme 2. Radioautography was carried out for about 5 days after which each of the radioactive spots on map (a) was eluted and counted; the counts/min in each spot are shown.

appear to contain any peptides in significant amounts since acid hydrolysis did not yield any predominant dansyl amino acid. It is likely that some of the sample was degraded during application of the samples to the silica gel and did not migrate.

In contrast to the unprotected enzyme, radioautographs of maps of the protected enzyme showed only a single radioactive peptide near the origin (Fig. 6b). This spot was in the same position as one of the major spots on the radioautographs of the unprotected enzyme. It therefore appears that only one group is concerned with the inactivation of both enzymic functions by iodoacetamide and their protection by prephenate. Amino acid analysis of the carboxymethylated protein (unpublished work) showed almost all the radioactivity to be associated with carboxymethylcysteine therefore the reactive group concerned is probably a cysteine residue.

DISCUSSION

The kinetics of the overall reaction catalysed by chorismate mutase–prephenate dehydrogenase were studied to determine whether the two enzymic reactions function independently when they are operating simultaneously. Two general criteria which may be used as characteristic of a system composed of totally independent partial reactions with similar turnover numbers are:

(1) The existence of a lag in the rate of formation of the final products.

(2) The accumulation of the product of the first reaction to a concentration comparable to that required to obtain a rate of product formation equal to the linear rate during the overall reaction, when the intermediate is used in an independent assay for the second reaction.

The validity of the above criteria has been confirmed by studies on chorismate mutase–prephenate dehydratase. There was a pronounced lag in the rate of formation of phenylpyruvate by the enzyme from *Salmonella typhimurium* and the rate of the

dehydratase reaction did not reach the rate of the mutase[8]. Experiments with chorismate mutase–prephenate dehydratase purified from *E. coli* showed that the amount of prephenate accumulating was equal to that required to demonstrate the same rate of phenylpyruvate formation with added prephenate (B. E. Davidson and T. A. A. Dopheide, personal communication).

The absence of a lag in the overall reaction catalysed by chorismate mutase–prephenate dehydrogenase from *A. aerogenes* indicates that the first criterion for a non-interacting system of sequential reactions is not satisfied for this enzyme system. The results obtained with chorismate mutase–prephenate dehydrogenase from *E. coli* also suggest the interdependence between the two functions. Although a distinct lag exists in the rate of formation of NADH during the incubation with chorismate and NAD^+, the small amount of prephenate which accumulates in the reaction mixture cannot account for the observed rate of the dehydrogenase reaction. Furthermore the rate of the dehydrogenase reaction soon becomes equal to that of the mutase. The prephenate concentration at the linear stage is about two orders of magnitude lower than that yielding the same rate of NADH formation during the assay for prephenate dehydrogenase activity alone. Thus the second criterion for independent sites mentioned above is not satisfied and this indicates that some interaction occurs between the two functions during the overall reaction.

It is possible that the mutase and dehydrogenase sites are so arranged as to permit direct transfer of the prephenate from the mutase to the dehydrogenase site, in which case prephenate would remain enzyme-bound during the overall reaction. Alternatively the affinity for prephenate at the dehydrogenase site may be markedly increased but prephenate not remain enzyme-bound, during the overall reaction. The absence of a lag in the overall reaction catalysed by the enzyme from *A. aerogenes* favours the model involving enzyme-bound prephenate since it suggests that no prephenate need accumulate in the reaction mixture prior to the operation of the dehydrogenase reaction at maximum velocity. The competitive inhibition of chorismate mutase by prephenate and the close similarity between the apparent K_m for prephenate and its K_i in the mutase reaction indicate that the two catalytic sites might share a common binding site for prephenate. Such an arrangement would clearly favour direct transfer of the prephenate between the two functions. Evidence against the model with enzyme-bound prephenate is given by the lag observed in the overall reaction catalysed by the enzyme from *E. coli*, but the amount of prephenate accumulating during the lag is very small. The lag may represent an activation period during which the enzyme assumes its most active form for the dehydrogenase reaction, possibly as an enzyme–prephenate complex, since the small amount of "free" prephenate formed could be that stoichiometrically bound to the enzyme.

The aim of the protein-modification studies was to determine the proximity of the mutase and dehydrogenase sites. Differential inactivation of the two functions by any of several modifying reagents was not obtained and prephenate was equally effective in protecting both activities against the reagents used. Such results are clearly consistent with an arrangement in which the two sites are close to one another or have components in common.

Further evidence for the hypothesis that the mutase and dehydrogenase sites are close to one another, or overlap, on the enzyme molecule was obtained from the radioautographs of tryptic peptide maps of enzyme labelled with radioactive iodo-

acetamide. Peptide maps of the enzyme treated in such a manner as to introduce radioactivity into only those residues protected by prephenate, contained only one radioactive peptide. Thus the protection, by prephenate, of both enzymic functions against inactivation by iodoacetamide seems to be the result of protection of a single reactive residue in the enzyme.

The observation that prephenate is an effective inhibitor of the chorismate mutase activity of chorismate mutase–prephenate dehydrogenase allows a possible rationalization of the finding that tyrosine inhibits only the prephenate dehydrogenase activity of purified chorismate mutase–prephenate dehydrogenase from *E. coli* or *A. aerogenes* rather than the chorismate mutase activity which is the first specific reaction on the tyrosine pathway[15,10,12] (see also ref. 16). Thus it is possible that tyrosine exerts a form of sequential feed-back inhibition causing the accumulation of prephenate which, in turn, inhibits chorismate mutase activity.

REFERENCES

1 G. L. E. KOCH, D. C. SHAW AND F. GIBSON, *Biochim. Biophys. Acta*, 212 (1970) 387.
2 G. L. E. KOCH, D. C. SHAW AND F. GIBSON, *Biochim. Biophys. Acta*, 229 (1971) 805.
3 A. GINSBURG AND E. R. STADTMAN, *Annu. Rev. Biochem.*, 39 (1970) 463.
4 J. YOURNO, T. KOHNO AND J. R. ROTH, *Nature*, 228 (1970) 820.
5 E. ADAMS, *J. Biol. Chem.*, 217 (1955) 325.
6 P. TRUFFA-BACHI, R. VAN RAPENBUSCH, J. JANIN, C. GROS AND G. N. COHEN, *Eur. J. Biochem.*, 7 (1969) 401.
7 T. E. CREIGHTON, *Biochem .J.*, 120 (1970) 699.
8 J. C. SCHMIT AND H. ZALKIN, *Biochemistry*, 8 (1969) 174.
9 F. GIBSON, *Biochem. Prep.*, 12 (1968) 94.
10 G. L. E. KOCH, D. C. SHAW AND F. GIBSON, *Biochim. Biophys. Acta*, 212 (1970) 375.
11 H. FRAENKEL-CONRAT, J. I. HARRIS AHD A. L. LEVY, *Methods Biochem. Anal.*, 2 (1955) 359.
12 G. L. E. KOCH, D. C. SHAW AND F. GIBSON, *Biochim. Biophys. Acta*, 229 (1971) 795.
13 J. M. EDWARDS AND L. M. JACKMAN, *Aust. J. Chem.*, 18 (1965) 1227.
14 R. M. C. DAWSON, D. C. ELLIOTT, W. H. ELLIOTT AND K. M. JONES, *Data for Biochemical Research*, Oxford University Press, London, 2nd ed., 1969, p. 196.
15 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 100 (1965) 76.
16 J. PITTARD AND F. GIBSON, *Current Topics in Cellular Regulation*, Vol. 2, Academic Press, New York, 1970, p. 53.