Reaction of Cytoplasmic Aspartate Aminotransferase with Tetranitromethane†

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ABSTRACT: The reaction of cytoplasmic aspartate aminotransferase with tetranitromethane has been investigated using the α subform of the pig heart enzyme. Tetranitromethane rapidly oxidizes two thiol groups (groups I and II) of either subunit of the dimeric enzyme. Concomitantly, enzymatic activity is decreased to ~60% of the original value. Blocking of groups I and II with *N*-ethylmaleimide precludes the initial inactivation by $C(NO_2)_4$. An additional thiol group (group III) is oxidized syncatalytically, *e.g.*, in the presence of the substrate pair glutamate and α-ketoglutarate. In parallel, enzymatic activity decreases to less than 5%. The enzyme derivative in which thiol group III has been cyanylated and which exhibits ~60% of the initial activity is not susceptible to syncatalytic inactivation. The remaining two thiol groups per subunit (groups IV and V) are unreactive toward tetranitromethane in the nondenatured protein. The analogy of the reactions of the thiol groups of aspartate aminotransferase with tetranitromethane and selective thiol reagents (*cf.* Birchmeier, W., Wilson, K. J., and Christen, P. (1973), *J. Biol. Chem. 248*, 1751) has been established by analysis of the CNBr peptides of the modified enzymes. In particular, with tetranitromethane the same cysteinyl residue is syncatalytically modified as with 5,5′-dithiobis(2-nitrobenzoic acid) and with *N*-ethylmaleimide, *i.e.*, cysteinyl residue 390 in the C-terminal region of the polypeptide chain. In the presence of substrates at pH 7.5, tetranitromethane nitrates up to 1 equiv of tyrosyl residues per enzyme subunit. Analysis of the CNBr peptides has revealed that nitration as compared to the thiol group modification is less selective; only half of the nitrotyrosine content is found in one particular CNBr fragment. Comparable tyrosyl nitration at pH 7.5 occurs also in the absence of substrates provided that thiol group III has been preoxidized by tetranitromethane at pH <6. However, nitration does not occur in the active enzyme derivative in which thiol group III has been cyanylated by cyanide. Thus, the oxidation of thiol group III represents the actual syncatalytic modification of cytoplasmic aspartate aminotransferase by tetranitromethane. Tyrosyl nitration appears to be a secondary reaction facilitated by oxidation of thiol group III.

Cytoplasmic aspartate aminotransferase is rapidly inactivated by $C(NO_2)_4$ provided that the reaction is carried out syncatalytically, *i.e.*, in the presence of the substrate pair glutamate and α-ketoglutarate (Christen and Riordan, 1970). One particular covalent enzyme–substrate intermediate of the transamination reaction appears to be markedly more susceptible toward modification than the free enzyme. The decrease in enzymatic activity upon reaction with $C(NO_2)_4$ was originally ascribed to tyrosyl nitration.

Recently we have reported similar syncatalytic inactivation of cytoplasmic aspartate aminotransferase by selective thiol reagents (Birchmeier and Christen, 1971). The inactivation is caused by modification of one particular sulfhydryl group out of a total of five on either subunit of the dimeric enzyme. This group (designated as thiol group III) has been identified in the primary structure of the polypeptide chain (Birchmeier *et al.*, 1972). It is thought to be located near the active site of the enzyme but not to be directly involved in catalysis, however, since the enzyme derivative which has been S-cyanylated at group III retains most of its activity. Two other sulfhydryl groups of each monomer (groups I and II) are more reactive by several orders of magnitude than group III; the remaining two groups (groups VI and V) are unreactive in the native enzyme (Birchmeier *et al.*, 1973). In view of the close similarity of the reactions of cytoplasmic aspartate aminotransferase with $C(NO_2)_4$ and with selective thiol reagents the syncatalytic reaction with $C(NO_2)_4$ was reinvestigated. The present paper demonstrates the occurrence of both thiol group *and* tyrosyl modifications and elucidates the interdependence of the two types of reactions.

Materials and Methods

Enzymes. The α subform of cytoplasmic aspartate aminotransferase (EC 2.6.1.1) was isolated from pig heart according to the procedure of Banks *et al.* (1968) as modified in our laboratory (Zaoralek and Christen, manuscript to be published). The concentration of the enzyme was determined spectrophotometrically using a molar absorptivity of the monomer $\epsilon_{280} = 7.0 \times 10^{-4}$ M^{-1} cm^{-1}. The specific activity was ~350 units/mg when assayed according to Karmen (1955). The pyridoxamine form of the enzyme was prepared by addition of 5 mM cysteine sulfinate (Jenkins and D'Ari, 1966); conversion to the pyridoxal form was performed by addition of 20 mM α-ketoglutarate; excess substrates were removed on a Sephadex G-25 column. Malate dehydrogenase (EC 1.1.37) was purchased from Boehringer.

Chemicals. L-Glutamic acid, L-aspartic acid, α-ketoglutaric acid, $C(NO_2)_4$, 2-mercaptoethanol, and Nbs_2 were from Fluka; L-cysteinesulfinic acid and α-methyl-DL-aspartic acid were from Sigma. Pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were from Merck; NADH was from Boehringer. [2,3-^{14}C]MalNEt was purchased from the Radiochem-

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[1] The abbreviations used are: $C(NO_2)_4$, tetranitromethane; MalNEt, *N*-ethylmaleimide; NbS_2, 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE I: Reaction of Cytoplasmic Aspartate Aminotransferase with $C(NO_2)_4$. Residual Enzymatic Activity and Degree of Thiol Group and Tyrosyl Modification.[a]

Reaction Conditions	% Initial Act.	Thiol Groups (mol/mol of Monomer)	Nitrotyrosyl Residues (mol/mol of Monomer)
Native pyridoxal enzyme	100	5.0	
+ $C(NO_2)_4$, no substrates			
pH 5.6	65	2.9[b]	0.03
pH 7.5	67	2.7[b]	0.24
+ $C(NO_2)_4$, Glu + α-ketoglutarate			
pH 5.6	15[c]	1.9	0.11
pH 7.5	3[c]	1.8	1.0[d]

[a] Reaction conditions were as in Figure 1. After a reaction time of 60 min the enzyme was separated from low molecular compounds by gel filtration on Sephadex G-25 columns equilibrated with the same buffer that was used for the modification reaction. Enzymatic activity and nitrotyrosyl content were determined as described under Materials and Methods. Thiol groups were measured using NbS_2 (Ellman, 1959) as described previously (Birchmeier *et al.*, 1973). The addition of 2-mercaptoethanol (20 mM) to the modified enzyme derivatives did not increase enzymatic activity >5%. Amino acid analyses of the enzyme treated with $C(NO_2)_4$ in the presence and absence of the substrate pair at pH 7.5 revealed no loss of histidyl or methionyl residues (residues per monomer in native enzyme, 7.7 ± 0.2 His and 4.8 ± 0.2 Met; in enzyme treated with $C(NO_2)_4$ in the presence of substrates, 7.5 ± 0.3 His and 5.2 ± 0.2 Met). [b] The loss of 2 equiv of thiol groups is already apparent after a reaction time of only 5 min. [c] Spectroscopic analysis of the derivative syncatalytically inactivated at pH 5.6 shows full interconvertibility between the pyridoxal and the pyridoxamine forms upon addition of the appropriate substrates. In contrast, the derivative modified at pH 7.5 appears to be irreversibly trapped in the pyridoxamine form. [d] Approximately the same degree of nitration is also obtained with higher (up to threefold) $C(NO_2)_4$ concentrations. A small pH-independent increase in absorbance between 400 nm and the protein absorption band which was observed after removal of the coenzyme may reflect modification of tryptophanyl residues (*cf.* Sokolovsky *et al.*, 1970).

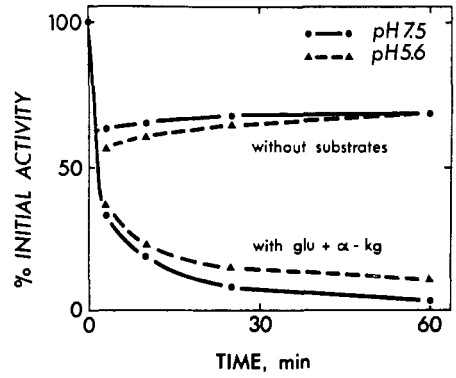

FIGURE 1: Inactivation of cytoplasmic aspartate aminotransferase by $C(NO_2)_4$ at pH 7.5 and 5.6. Enzyme (0.07 mM monomer) was incubated with 3.0 mM $C(NO_2)_4$ at 25° either in the pyridoxal form or in the presence of 70 mM glutamate (glu) plus 2 mM α-ketoglutarate (α-kg). The buffers used were 0.05 M Tris-Cl (pH 7.5) or 0.05 M Tris-acetate (pH 5.6). At the indicated times aliquots were analyzed for aminotransferase activity.

ical Centre, Amersham. Guanidine hydrochloride (Ultra Pure) was from Mann; CNBr was from Pierce.

Chemical Modifications. Modifications of thiol groups I and II with MalNEt and S-cyanylation of thiol group III were performed as described elsewhere (Birchmeier *et al.*, 1973). Modifications with $C(NO_2)_4$ were carried out with 3.0 mM reagent (added as a 0.3 M solution in ethanol) in 0.1 M sodium phosphate (pH 7.5) or in 0.1 M sodium acetate (pH 5.3) in either the absence or the presence of 70 mM glutamate and 2 mM α-ketoglutarate. Excess reagents and substrates were removed by gel filtration on Sephadex G-25 columns equilibrated in 0.1 M phosphate (pH 7.5). Denatured enzyme was freed from reagents on Sephadex G-25 columns equilibrated in 70% formic acid. All modification experiments were carried out at room temperature unless stated otherwise. The nitrotyrosyl content was determined spectrophotometrically at pH 8.5 following conversion of the enzyme derivatives to the pyridoxamine form and assuming $\epsilon_{428} = 4100\ M^{-1}\ cm^{-1}$ (Sokolovsky *et al.*, 1966). The measurements were corrected for the weak absorbance of the unmodified enzyme at this wavelength. The nitrotyrosyl content of the denatured enzyme and of the CNBr fragments was determined after solubilization of lyophilized material in 2% NH_4OH–0.5% sodium dodecyl sulfate and nitrotyrosine was identified by the change in the absorption spectrum upon addition of formic acid to a final concentration of 10%.

CNBr cleavage of enzyme derivatives modified at all five thiol groups was carried out according to the procedure of Steers *et al.* (1965). The resulting fragments were fractionated on a column of Sephadex G-75 (1.5 × 150 cm) equilibrated with 10% formic acid (Watanabe and Wada, 1971). Absorption spectra were recorded at room temperature on a Cary 15 spectrophotometer; absorbance at discrete wavelengths was determined with a Unicam SP 1800 spectrophotometer. Radioactivity was measured in a TriCarb scintillation spectrometer, Model 3080, as described previously (Birchmeier *et al.*, 1973). Amino acid analyses were performed in triplicate with a Beckman 120B amino acid analyzer following hydrolysis of the samples in 6 N HCl for 22, 44, and 72 hr at 110° in evacuated tubes.

Results

Syncatalytic Modification of Cytoplasmic Aspartate Aminotransferase by $C(NO_2)_4$. Addition of 3 mM $C(NO_2)_4$ to the pyridoxal form of the enzyme results in a rapid initial decrease of enzymatic activity to ~60% (Figure 1). However, in the presence of the substrate pair glutamate and α-ketoglutarate, both at pH 5.6 and 7.5, enzymatic activity decreases further to a few per cent of the original value. The initial rapid decrease in enzymatic activity is accompanied by modification of two thiol groups (groups I and II, see below) out of a total of five per enzyme monomer (Table I). The slower time-dependent loss of activity which occurs in the presence of glutamate and α-ketoglutarate correlates with the modification of

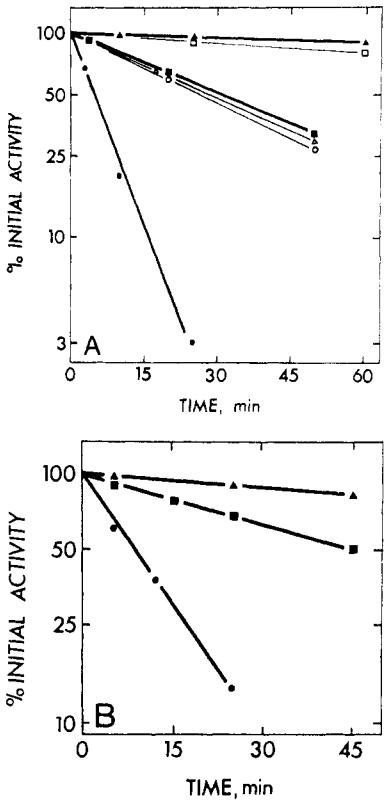

FIGURE 2: Dependence of the rate of inactivation on the functional state of the enzyme. The enzyme used was premodified at thiol groups I and II with MalNEt resulting in a 1.25-fold increase in enzymatic activity (Birchmeier *et al.*, 1973). This value was taken as 100% in this graph. The reaction was performed at 25° with 0.06 mM enzyme (monomer) and 3.0 mM $C(NO_2)_4$: (A) in 0.1 M sodium phosphate (pH 7.5); (B) in 0.1 M sodium acetate (pH 5.3); (▲) pyridoxal enzyme in the absence of substrates; (■) pyridoxamine enzyme in the absence of substrates; (●) enzyme in the presence of 70 mM glutamate and 2 mM α-ketoglutarate; (□) pyridoxal enzyme in the presence of 2 mM α-ketoglutarate; (○) pyridoxal enzyme in the presence of 20 mM α-methyl aspartate; (△) pyridoxamine enzyme in the presence of 70 mM glutamate.

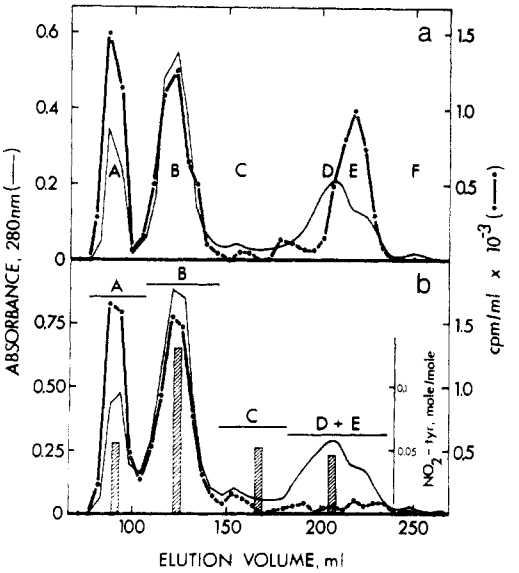

FIGURE 3: Localization of the $C(NO_2)_4$-modified cysteinyl and tyrosyl residues in the CNBr peptides; chromatography on a Sephadex G-75 column in 10% formic acid. The enzyme was premodified at thiol groups I and II with MalNEt and then modified with $C(NO_2)_4$ at pH 7.5 for 30 min in either the absence or the presence of glutamate and α-ketoglutarate. Subsequently, the thiol groups that had not been modified by $C(NO_2)_4$ were alkylated with 2 mM [^{14}C]MalNEt (7.0×10^4 cpm/μmol) for 2 hr in 6 M guanidine-HCl–0.1 M sodium phosphate (pH 7.5): (a) $C(NO_2)_4$ reaction carried out in the absence of substrates (enzymatic activity 85%; thiol groups 2.9 mol/mol of monomer; [^{14}C]MalNEt incorporated 2.6 mol/mol of monomer; nitrotyrosyl content 0.1 mol/mol of monomer); (b) $C(NO_2)_4$ reaction carried out in the presence of glutamate and α-ketoglutarate (enzymatic activity 0.7%; thiol groups 1.9 mol/mol of monomer; [^{14}C]MalNEt incorporated 1.5 mol/mol of monomer; nitrotyrosyl content 0.32 mol/mol of monomer). The nitrotyrosyl content was determined in the pooled fractions designated by the solid bars.

a third thiol group (group III, see below). In contrast to thiol oxidation the degree of tyrosyl modification does not parallel inactivation; significant nitration, 1 equiv of tyrosyl residues per enzyme monomer, occurs only at pH 7.5 in the presence of the substrate pair.[2]

Modification of thiol groups I and II with MalNEt (Birchmeier *et al.*, 1973) results in an increase of enzymatic activity to 125% of the original value and prevents the rapid initial inactivation by $C(NO_2)_4$ (Figure 2). This enzyme derivative has been used to study the effect of the functional state of the enzyme on the rate of inactivation. The slowest inactivation rates at pH 7.5 are observed with the free pyridoxal form of the enzyme (pseudo-first-order rate constant $k'_{inact} = 1.8 \times 10^{-3}$ min^{-1}) and with the pyridoxal form of the enzyme in the presence of the competitive inhibitor α-ketoglutarate ($k'_{inact} = 3.4 \times 10^{-3}$ min^{-1}). A 15 times higher rate is measured with the pyridoxal form in the presence of the substrate analog α-methyl aspartate ($k'_{inact} = 2.7 \times 10^{-2}$ min^{-1}). In the presence of glutamate and α-ketoglutarate the rate of inactivation is increased nearly 100 times ($k'_{inact} = 1.4 \times 10^{-1}$ min^{-1}). The free pyridoxamine enzyme and its complex with glutamate exhibit an intermediate behavior ($k'_{inact} = 1.5 \times 10^{-2}$ min^{-1} and 2.2×10^{-2} min^{-1}, respectively). Importantly, quite similar rates are obtained when the reactions with $C(NO_2)_4$ is carried out at pH 5.3 (Figure 2B) where oxidation of thiol group III occurs with minimal nitration of tyrosyl residues (Table I).

Localization of the Modified Residues in the CNBr Peptides. The thiol groups remaining unmodified during reaction of the enzyme with $C(NO_2)_4$ were blocked with [^{14}C]MalNEt in 6 M guanidine-HCl. The CNBr fragments of these derivatives were chromatographed on a column of Sephadex G-75 (Figure 3). In the derivative premodified with $C(NO_2)_4$ in the *absence* of the substrate pair the 3 equiv of the incorporated radioactive label was found distributed in equal portions in fragments A, B, and E (Figure 3a). The 2 equiv of thiol groups remaining unmodified by $C(NO_2)_4$ in the *presence* of the substrate pair was located exclusively in peptides A and B (Figure 2B). Apparently, the thiol group susceptible to syncatalytic modification by $C(NO_2)_4$ is the one of peptide E (group III, *cf.* Birchmeier *et al.*, 1973). The nitrotyrosyl residues of the syncatalytically modified enzyme were found to be distributed in several CNBr fragments, the highest nitrotyrosyl

[2] With the commercially available enzyme the presence of substrates induced nitration of only 0.5 additional equiv of tyrosyl residues per monomer when compared with nitration in the absence of substrates (Christen and Riordan, 1970). In this previous report tyrosyl nitration was considered the only effect of $C(NO_2)_4$ under the conditions of syncatalytic modification. The titrations of thiol groups with *p*-chloromercuribenzoate (Boyer, 1954) which were performed using the nondenatured enzyme derivative failed to detect the loss of thiol group III apparently due to the low reactivity of this group.

TABLE II: Reaction of $C(NO_2)_4$ with the Enzyme Derivative Modified at Thiol Group III with Cyanide. Effect on Enzymatic Activity and Tyrosyl Nitration.[a]

S-Cyanylated Enzyme	% Initial Act. 15 min	% Initial Act. 60 min	Nitrotyrosyl Content after 60 min of Reaction (mol/mol of Monomer)
Pyridoxal form	98	93	0.18
+ Glu + α-keto-glutarate	96	85	0.14
Pyridoxamine form	93	76	0.22

[a] Enzyme (0.06 mM monomer) modified at thiol groups I and II with MalNEt and at group III with cyanide (sp act. 260 units/mg) was incubated with $C(NO_2)_4$ at pH 7.5 under the conditions of Figure 2. After 60 min the reaction was quenched by gel filtration and the nitrotyrosyl content was measured as described under Materials and Methods.

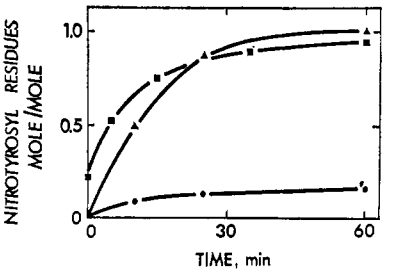

FIGURE 4: Time course of tyrosyl nitration in cytoplasmic aspartate aminotransferase. $C(NO_2)_4$ (3.0 mM) was added to enzyme (0.08 mM monomer) in 0.05 M sodium phosphate (pH 7.5) at 25°. The nitrotyrosyl content was determined as described under Materials and Methods after quenching the reaction at the indicated times by rapid gel filtration: (●) native pyridoxal enzyme in the absence of substrates; (▲) native enzyme in the presence of glutamate (70 mM) plus α-ketoglutarate (2 mM); (■) in this enzyme derivative thiol groups I, II, and III were modified in a prior reaction with $C(NO_2)_4$ at pH 5.3 in the presence of glutamate and α-ketoglutarate (*cf.* Table I); the enzyme derivative was then converted to the pyridoxal form and freed from small molecular compounds by gel filtration; the present reaction with $C(NO_2)_4$, at pH 7.5, was carried out in the absence of substrates.

content, approximately half of the total, being recovered in peptide B.

Dependence of Tyrosyl Nitration on Preceding Thiol Oxidation. The nitration reaction in the presence of glutamate and α-ketoglutarate at pH 7.5 reaches a maximum value of approximately 1 equiv of nitrotyrosyl residues per enzyme monomer (Figure 4). However, the same degree of nitration is obtained also with the pyridoxal form of the enzyme in the *absence* of substrates provided that thiol group III has been premodified syncatalytically by $C(NO_2)_4$ at pH 5.3.[3] Apparently, tyrosyl modification is a consequence of the preceding oxidation of thiol group III rather than of the presence of substrates. Results obtained with an enzyme derivative in which sulfhydryl group III has been cyanylated and which exhibits 60% of the initial activity (Birchmeier *et al.*, 1973) are consistent with this conclusion. When the cyanylated derivative was exposed to $C(NO_2)_4$ under the conditions used for syncatalytic modification (Table II) marked inactivation and nitration of tyrosyl residues did not occur either in the pyridoxal or pyridoxamine form or in the presence of glutamate and α-ketoglutarate.

Discussion

$C(NO_2)_4$ has been widely used in chemical modification studies on the involvement of tyrosyl residues in the structure and function of peptides and proteins (Sokolovsky *et al.*, 1966; Riordan and Sokolovsky, 1971). Besides forming 3-nitrotyrosyl residues the reagent reacts also with cysteinyl side chains producing mainly disulfide or sulfinate derivatives (Riordan and Christen, 1968; Sokolovsky *et al.*, 1969). Under certain circumstances the reaction of $C(NO_2)_4$ with histidyl, tryptophanyl, and methionyl residues, as well as inter- and intramolecular cross-links of tyrosyl residues have been reported (*cf.* Riordan and Vallee, 1972).

In the present study the reactions of $C(NO_2)_4$ with both sulfhydryl groups and tyrosyl residues of cytoplasmic aspartate aminotransferase were examined. Two thiol groups out of a total of five per enzyme monomer are the residues most susceptible to modification by $C(NO_2)_4$ (Table I). The same two groups are also quite reactive toward sulfhydryl reagents such as Nbs_2 or MalNEt and are located apparently on the surface of the enzyme molecule (Birchmeier *et al.*, 1973). The rapid initial decrease in enzymatic activity to 60% of the original value upon addition of $C(NO_2)_4$ is thought to be due to the oxidation of at least one of these two thiol groups. Inactivation does not occur when the enzyme has been premodified at thiol groups I and II by MalNEt (Figure 2) or by maleate according to Turano *et al.* (1964) (Birchmeier, W., and Christen, P., unpublished results). The initial decrease in activity was also not observed with the commercially available enzyme (Christen and Riordan, 1970) which was isolated in the presence of maleate as a protecting ligand (Jenkins *et al.*, 1959). Disulfides do not appear to be the product of the reaction of $C(NO_2)_4$ with thiol groups I and II. Only slight reactivation occurs upon addition of 2-mercaptoethanol to the modified enzyme. Apparently, the location of groups I and II in the three-dimensional structure of the enzyme precludes intra- or intermolecular disulfide formation. Consistent with this conclusion modification of the native enzyme with Nbs_2 results in incorporation of 2 equiv of thionitrobenzoate at sulfhydryl groups I and II (Birchmeier *et al.*, 1973) though a cysteinyl-thionitrobenzoate disulfide would readily undergo disulfide exchange with any neighboring free thiol group sterically available (*cf.* Connellan and Folk, 1969; Flashner *et al.*, 1972).

Additional reactions of $C(NO_2)_4$ with residues other than cysteinyl I and II depend on the functional state of the enzyme. As shown previously, the presence of the substrate pair glutamate and α-ketoglutarate induces marked inactivation of the enzyme by $C(NO_2)_4$ (Christen and Riordan, 1970). The present data demonstrate that this inactivation which occurs when the enzyme molecules are engaged in covalent enzyme–substrate intermediates is accompanied by oxidation of a third thiol group per enzyme subunit (Table I). The double modification experiments indicate that this thiol group is the same one which is syncatalytically modified by selective thiol reagents (Figure 3). This group, designated as thiol group III, has been identified in the C-terminal CNBr fragment E from

[3] With an enzyme derivative premodified at thiol group III with MalNEt up to 2 equiv of tyrosyl residues per monomer was nitrated in the absence of substrates.

which the thiol-containing tryptic peptide has been isolated (Birchmeier *et al.*, 1972). Comparison of its amino acid sequence with the recently reported primary structure of cytoplasmic aspartate aminotransferase (Ovchinnikov *et al.*, 1973) has indicated that thiol group III is located in the 390th position of the polypeptide chain. These findings have lately been confirmed by Torchinsky *et al.* (1972). Thiol groups IV and V which are unreactive toward MalNEt and Nbs_2 in the native enzyme maintain their buried state also toward $C(NO_2)_4$. Apparently, the classification of the five sulfhydryl groups of cytoplasmic aspartate aminotransferase based on their reactivity toward Nbs_2 and MalNEt (Birchmeier *et al.*, 1973) applies also to their reaction with $C(NO_2)_4$.

The reactivity of thiol group III as reflected by the rate of enzyme inactivation by $C(NO_2)_4$ changes from one enzyme–substrate intermediate to the other (Figure 2). These alterations in reactivity have been previously examined in detail using MalNEt as the modifying reagent and have been interpreted to reflect conformational changes of the enzyme–coenzyme–substrate complex in the course of catalysis (Birchmeier *et al.*, 1973). Maximum reactivity of sulfhydryl group III toward MalNEt has been observed in the ketimine intermediate. Apparently, it is also the intermediate most reactive toward $C(NO_2)_4$ since the highest rate of inactivation is measured in the presence of the substrate pair and not of the competitive inhibitors or of the substrate analog α-methyl aspartate which forms only the aldimine intermediate. The susceptibility of the semiquinoide intermediate accumulated in the presence of the substrate analog *erythro*-β-hydroxy aspartate (Jenkins, 1964) was not examined in the present study because $C(NO_2)_4$, like other oxidants, has been found to be consumed by reaction with the carbanionic centers of this intermediate (Shlyapnikov and Karpeisky, 1969; Healy and Christen, 1973).

The occurrence of both sulfhydryl *and* tyrosyl modifications raises the question as to which one is responsible for the decrease in enzymatic activity. The following experimental data indicate that modification of sulfhydryl group III is the primary structural correlate of the syncatalytic inactivation. Inactivation by $C(NO_2)_4$ was also found at pH <6, where oxidation of thiol group III occurs without significant tyrosyl nitration; cyanylation of group III with cyanide which does not impair enzymatic activity (Birchmeier *et al.*, 1973) prevents inactivation by $C(NO_2)_4$. The rate of inactivation at pH 7.5, particularly in the enzyme derivative premodified at thiol group I and II with MalNEt, is considerably faster than the rate of tyrosyl nitration (*cf.* legend of Figure 3b). Most likely the oxidation of sulfhydryl group III results in an alteration of the conformational structure of the enzyme which markedly reduces the enzymatic activity and renders possible the nitration of otherwise unreactive tyrosyl residues. Consistent with this view, modification of sulfhydryl group III with the small and uncharged cyano substituent neither impairs enzymatic activity (Birchmeier *et al.*, 1973) nor facilitates tyrosyl nitration (Table II).

All modifications of thiol group III examined including oxidation with $C(NO_2)_4$ at pH <6 (*cf.* Table I) do not completely abolish enzymatic activity; these enzyme derivatives are still fully interconvertible between the pyridoxal and the pyridoxamine forms indicating residual activity of each individual enzyme molecule (Birchmeier *et al.*, 1973). However, after modification with $C(NO_2)_4$ at pH 7.5, when (in addition to oxidation of sulfhydryl group III) tyrosyl residues are nitrated, the enzyme is irreversibly trapped in the pyridoxamine form (Christen and Riordan, 1970; Bocharov *et al.*, 1973). In contrast to thiol modification tyrosyl nitration is not residue specific; the tyrosyl residues nitrated out of a total of 12 per enzyme subunit (Doonan *et al.*, 1972; Ovchinnikov *et al.*, 1973) are distributed in different CNBr peptides (Figure 3). The main fraction is found in CNBr fragment B which constitutes the N-terminal region (residues 1–212; Birchmeier, W., and Christen, P., unpublished results) of the polypeptide chain. Polyanovsky *et al.* (1972) have recently analyzed the main nitrotyrosyl-containing tryptic peptide from syncatalytically modified enzyme. Apparently, the nitratable tyrosyl residue is located at the 40th position of the polypeptide chain (Ovchinnikov *et al.*, 1973). Though the present data indicate that tyrosyl nitration is a secondary event in the syncatalytic reaction with $C(NO_2)_4$, they do not exclude a possible functional role of tyrosyl residue 40. However, the elucidation of the function of this residue would seem to require improvement of the as yet limited residue specificity of the chemical modifications employed.

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A Chemical Model for Thymidylate Synthetase Catalysis†

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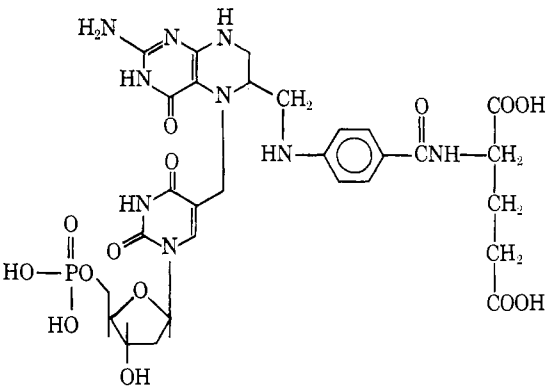
ABSTRACT: In the thymidylate synthetase catalyzed reductive methylation of 2′-deoxyuridine 5′-phosphate to give thymidine 5′-phosphate, 5-thymidylyltetrahydrofolic acid has been proposed as an intermediate which rearranges to give the observed products. However, an analog of the proposed intermediate, 5-thyminyltetrahydrofolic acid, was reported to be stable to air and did not undergo rearrangement to thymine when heated to 100° at pH 7 (Gupta, V. S., and Huennekens, F. M. (1967), *Biochemistry 6*, 2168). A chemical model is described in this paper which provides chemical precedence for the rearrangement of the proposed enzymatic intermediate. Heating 1,2-dihydro-*N*-thyminylquinoline to 200° under vacuum produced thymine and quinoline in 42 and 47% yields, respectively. The rearrangement could also be effected in refluxing solvents such as diglyme or water. When 1,2-dihydro-*N*-thyminylquinoline-*2,2-d*$_2$ was rearranged, thymine containing one deuterium in the methyl group was isolated. No exchange of the migrating deuterium with solvent occurred when the latter rearrangement was conducted in an aqueous medium. A crossover experiment using two deuterium-labeled model compounds provided evidence for both intramolecular and intermolecular rearrangements in these model compounds. In an extension of the model, 1,2,3,4-tetrahydro-*N*-thyminylquinoline was prepared in one step from uracil, formaldehyde, and 1,2,3,4-tetrahydroquinoline. This latter model compound produced thymine when heated to 250° under vacuum. This provides a chemical model in which uracil can be converted to thymine through a bridged intermediate similar to that proposed in the literature.

Thymidylate synthetase catalyzes the reductive methylation of dUMP[1] to give dTMP utilizing formaldehyde as the carbon source and H_4folate as the reducing agent (Friedkin, 1963; Blakely, 1967). The vital role of thymidylate synthetase in the biosynthesis of DNA has made it an attractive target enzyme in the chemotherapy of cancer (Hartmann and Heidelberger, 1961; Wolberg, 1969). Considerable interest has been focused upon determining the mechanism of this transformation which would greatly facilitate the rational design of inhibitors of the enzyme (Baker, 1967; Santi, 1967).

Extensive tritium labeling studies have shown that the hydrogen on C-6 of H_4folate is transferred exclusively to the methyl group of dTMP and does not exchange with the reaction medium during the transfer (Pastore and Friedkin, 1962; Lorenson *et al.*, 1967). A mechanism proposed by



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Friedkin which is in agreement with the labeling studies involves the formation of 5-thymidylyltetrahydrofolic acid (**1**) as an intermediate which would then undergo rearrangement *via* a 1,3-hydride shift to give the observed products (Friedkin and Kornberg, 1957; Wahba and Friedkin, 1962).

The finding that thymidylate synthetase catalyzes the exchange of the 5-H of dUMP with water and that this occurs at maximum velocity only when all the components of the enzymatic reaction are present has been interpreted as supporting such a two-step mechanism (Lomax and Greenberg, 1967). However, 5-thyminyltetrahydrofolic acid, an analog

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[1] Abbreviations used are: dUMP, 2′-deoxyuridine 5′-monophosphate; dTMP, thymidine 5′-monophosphate; H_4folate, tetrahydrofolic acid.