Functional Groups of Diphosphopyridine Nucleotide Linked Isocitrate Dehydrogenase from Bovine Heart. I. Studies of an Active Amino Group by Amidination, Arylation, Acetylation, and Carbamylation†

Chinan C. Fan and Gerhard W. E. Plaut*

ABSTRACT: DPN-linked isocitrate dehydrogenase is inactivated by a number of amino group specific reagents including trinitrobenzenesulfonate, KNCO, ethyl acetimidate, and ethyl benzylimidate. At pH 7.2, loss of activity corresponds to trinitrophenylation or carbamylation of a single amino group per subunit of enzyme. The pH–rate profiles of inactivation show that this amino group has a pK_a of 8. Increasing ionic strength inhibits the rate of inactivation by the sulfonate but does not alter the value of pK_a of the reactive group. The rate of inactivation is not linear with trinitrobenzenesulfonate concentration and evidence has been presented for formation of a dissociable enzyme–trinitrobenzenesulfonate complex which is converted subsequently to the covalently linked trinitrophenyl enzyme. The pK_a of the binding site for formation of this complex is different from that of the reactive group on the enzyme which is trinitrophenylated. Heterogeneity of the reactivity of the sulfonate with the 24 lysyl residues/subunit of enzyme has been observed at pH 7.2 and 25°. Protection against inactivation by all reagents has been obtained with manganese isocitrate, but not with free isocitrate.

Earlier studies have shown that DPN-linked isocitrate dehydrogenase from heart and liver is inhibited by low concentrations of *p*-mercuribenzoate, *p*-mercuribenzenesulfonate, and 5,5′-dithiobis(2-nitrobenzoate) (Chen *et al.*, 1964; Plaut and Aogaichi, 1968; Plaut, 1970). Protection against inhibition by these reagents was obtained with a combination of activating divalent cation (Mn^{2+} or Mg^{2+}) and D-*threo*-isocitrate (but not the L isomer); however, the addition singly of divalent metal ions, isocitrate, ADP, and DPN^+ or DPNH did not afford protection. The sensitivity to these sulfhdryl group binding reagents and the recent demonstration that a divalent metal ion isocitrate complex, *viz.*, magnesium isocitrate, is the true substrate of the enzyme (Plaut *et al.*, 1973) suggest that a reactive sulfhydryl group(s) may be involved at the active site of the enzyme.

In order to define more closely the participation of other amino acid residues in the binding of substrates and activators to the enzyme and the participation of such sites in catalysis and enzyme regulation a systematic study of the effects of group-specific reagents was undertaken.

Preliminary studies of DPN-linked isocitrate dehydrogenase from bovine heart indicated inhibition of activity by KNCO at neutral pH.[1] Neutral or weakly alkaline conditions have been reported to favor the carbamylation of amino groups by cyanate (Stark, 1967). In the present report the effect on activity and the stoichiometry of binding of group-specific reagents which can combine irreversibly with amino groups on the enzyme is described. In the accompanying report (Fan and Plaut, 1974) the stoichiometry of interaction of a number of aldehydes with a specific amino group leading to substrate-reversible aldoxylidene formation and enzyme inactivation is discussed.

Experimental Procedures

Materials. DPN-linked isocitrate dehydrogenase was purified from lyophilized mitochondria from bovine heart by the method of Giorgio *et al.* (1971). It showed a single band in polyacrylamide gel disc electrophoresis and had a specific activity of 28,000 nmol of DPN^+ reduced per min per mg of protein at 25° under the conditions of assay reported previously (Giorgio *et al.*, 1971).

Ethyl acetimidate hydrochloride and ethyl benzylimidate hydrochloride were prepred by the method of Pinner (1883) as modified by McElvain and Nelson (1942) by treating an ether solution of ethanol and acetonitrile or ethanol and phenylacetonitrile with HCl gas.

Potassium cyanate was purchased from Eastman and potassium [^{14}C]cyanate from ICN. 2,4,6-Trinitrobenzenesulfonic acid (sodium salt) was obtained from Sigma, *N*-acetylimidazole from Eastman, and 5,5′-dithiobis(2-nitrobenzoic acid) from Aldrich.

Inorganic salts were analytical reagent grade from Mallinckrodt Chemical Works. ADP, DPN^+, DPNH, Hepes,[2] and isocitric lactone were from Sigma. Before use isocitric lactone was hydrolyzed in alkali as described previously (Plaut, 1969).

Standard Assay. Enzyme activity was determined as described previously (Giorgio *et al.*, 1971) in a reaction mixture containing 166 mM NaHepes at pH 7.2, 5.3 mM DL-isocitrate, 1.3 mM $MnCl_2$, 0.33 mM DPN^+, and 0.67 mM ADP in a final volume of 1.0 ml. Reactions were initiated by the addition of 10 μl or less of appropriately diluted native or modified enzyme. Initial reation rates at 25° were estimated from the density change (at 340 nm) with time from the initial linear

† From the Department of Biochemistry, School of Medicine, Temple University, Philadelphia, Pennsylvania 19140. *Received August 16, 1973.* Supported in part by Research Grant AM 15404 from the National Institute of Arthritis, Metabolic and Digestive Diseases, U. S. Public Health Service. A preliminary report of these studies has appeared (Fan and Plaut, 1973).

[1] A. Yip and G. W. E. Plaut, unpublished observations.

[2] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

portion of the record. Velocities were expressed as formation of nanomoles of DPNH per milliliter of reaction mixture per minute at 25° (Plaut, 1969).

Enzyme Protein. Protein concentration was determined spectrophotometrically at 278 nm at neutral pH. A solution of the enzyme which contains 1% protein by the method of Lowry *et al.* (1951) has an absorbance of 5.3 cm^{-1}.

Enzyme concentration is expressed on the basis of a subunit molecular weight of 42,000 daltons (Giorgio *et al.*, 1971).

Enzyme Modification. Enzyme (8 μM unless specified otherwise) was treated with various reagents under the conditions described later. Protection against inhibition was studied by adding potentially protective substances to the enzyme before addition of modifying agents. Incubation under identical conditions in the absence of modifiers served as controls. After the addition of modifying agents, aliquots of 10 μl or less were taken from the reaction mixtures at appropriate time intervals and activity was tested by the standard assay. Deviations from this procedure are indicated where appropriate. The rates of inactivation of enzyme activity have been expressed as pseudo-first-order rate constants (k_1). The extent of chemical modification of the enzyme protein was determined by the methods described later.

Amidination. Immediately before use, ethyl acetimidate·HCl or ethyl benzylimidate·HCl was dissolved in water and neutralized with NaOH. The amidinating reagents were added at a final concentration of 10 mM to solutions of the enzyme in a mixed buffer containing 50 mM sodium borate and 50 mM NaHepes at the values of pH indicated, the solutions were incubated at 23° for various periods of time and aliquots were tested for activity by the standard assay.

Carbamylation. Enzyme was incubated with 0.05–0.50 M potassium cyanate in 0.5 M NaHepes at pH 7.2 and 23°. The carbamylation was stopped after varying periods of incubation by the addition of excess lysine followed by extensive dialysis at 5° against 5 mM sodium phosphate buffer at pH 7.2 containing 20% glycerol and 0.1 mM 1,3-dithio-2-propanol. Aliquots of the dialyzed solutions were tested for activity or, when potassium [^{14}C]cyanate was used, for incorporation of radioactivity into protein by counting appropriate aliquots in a scintillation counter.

Trinitrophenylation. The dehydrogenase was incubated at 25° with a 1- to 1000-fold molecular excess of trinitrobenzenesulfonate over enzyme in the buffers specified in the text, tables, and figures. The modification reaction was stopped by the addition of β-mercaptoethanol or lysine in a 10- to 100-fold excess over the sulfonate present in the incubation mixture.

The treated preparations were dialyzed extensively against buffers containing 0.1% sodium dodecyl sulfate to remove free sulfonate and sulfite. The proteins were then precipitated with trichloroacetic acid; the residues were washed with 10% trichloroacetic acid and finally dissolved in 88% formic acid for the spectrophotometric determinations. The trinitrophenylated derivatives have absorption maxima at 345 and 420 nm. The molar extinction coefficient of 1.45×10^4 at 345 nm for ϵ-trinitrophenyl-α-acetyllysine (Okuyama and Satake, 1960) was used to estimate the extent of trinitrophenylation of the protein.

Sulfite, which is liberated during trinitrophenylation, changes the absorption characteristics of the trinitrophenylated group. However, Goldfarb (1966) and Coffee *et al.* (1971) have found that the absorbance of trinitrophenylated derivatives at 367 nm is independent of sulfite concentration. Under certain conditions the extent of modification was therefore determined without preliminary dialysis by direct spectrophotometric measurements at 367 nm with an appropriate reagent blank. The molecular extinction coefficient of 1.05×10^4 was used to estimate the amount of modification of protein.

Results

Trinitrophenylation

The enzyme is very susceptible to inhibition by trinitrobenzenesulfonate. The addition of five molecules of the sulfonate per subunit molecule of enzyme led to an 80% loss of activity in 2 hr. As the concentration of the sulfonate was increased, the pseudo-first-order rate constants of enzyme inactivation also increased (Figure 1).

Since trinitrobenzenesulfonate can react with both thiol and amino groups (Okuyama and Satake, 1960), it was important to establish whether or not the inactivation is attributable to interaction of the sulfonate with the thiol groups of the enzyme. The sulfhydryl content of the sulfonate modified enzyme was determined by the method of Ellman (1959) by difference spectroscopy at 412 nm with the trinitrophenylated protein serving as the blank. Modified enzyme which had been inactivated about 50–60% contained the same number of thiol groups as the enzyme before treatment.[3] These results suggest that inactivation by the sulfonate is caused by modification of one or more amino groups. However, the sulfhydryl content of the protein did decline when inactivation by the sulfonate exceeded 80% of the original activity, indicating that reaction of the sulfonate with amino groups as well as thiol groups can occur, albeit at different rates.

Effect of pH and Ionic Strength on Enzyme Inactivation. When the concentrations of trinitrobenzenesulfonate and enzyme were constant, a plot of pseudo-first-order rate constants of enzyme inactivation against pH yielded a sigmoid curve (Figure 2). If one assumes that the rate of reaction with the sulfonate is dependent on the concentration of the conjugate base form of a functional group on the enzyme, one can calculate from the inflection point of the curve that the value for this group is $pK_a = 7.95$.

Inactivation of the enzyme is markedly dependent on ionic strength. The effect of changes in NaCl concentration on the ratio of enzyme inactivation by 0.25 mM trinitrobenzenesulfonate at pH 7.2 was studied by varying the NaCl concentration from 0 to 1 M. The pseudo-first-order rate constant of inactivation was over fivefold larger in the absence of added NaCl than in the presence of 0.6 M NaCl at pH 7.2, and NaCl depressed the rate of inactivation between pH 6 and 8.8 (Figure 2). Nevertheless, the pK_a value for the reactive amino group obtained in the same buffer with and without NaCl was approximately the same for the sulfonate inactivation.

Protection by Substrate and Cofactors. As shown in Table I the rate of inactivation by trinitrobenzenesulfonate is markedly diminished by a combination of manganese and isocitrate while the equivalent concentration of isocitrate alone was ineffective. Mn^{2+} or DPNH when added singly also afforded substantial protection; however, each was less effective than

[3] C. C. Fan and G. W. E. Plaut, unpublished observations, 1972. Even though different batches of enzyme had essentially the same specific activities, they contained from 5.3 to 6 thiol groups per subunit. In view of this variability, the comparisons of the thiol content of the protein before and after modification by the sulfonate were always done with *identical* batches of enzyme preparations.

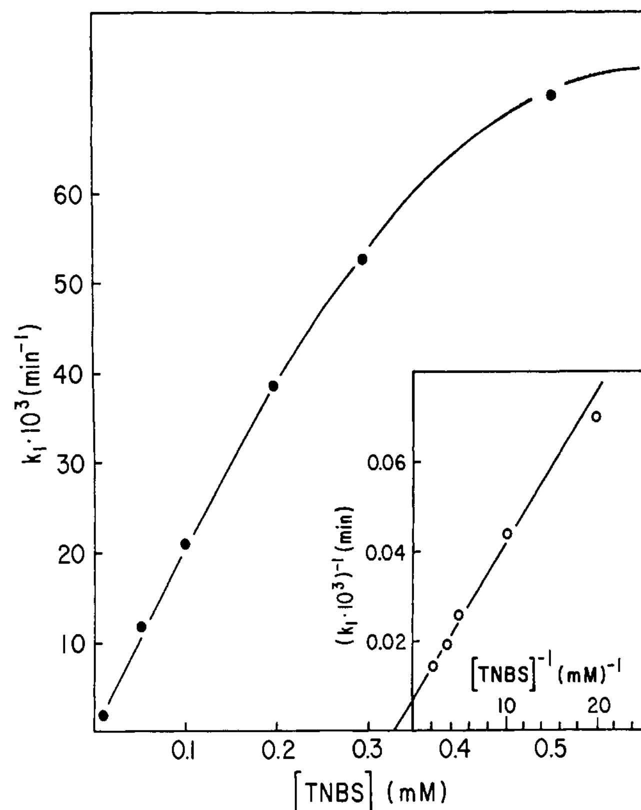
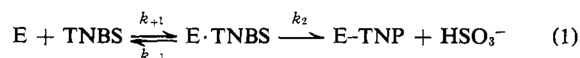


FIGURE 1: The effect of trinitrobenzenesulfonate (TNBS) concentration on enzyme inactivation. Enzyme (0.01 mM) was incubated with 0.01–0.50 mM TNBS in 5 mM sodium phosphate buffer containing 20% glycerol at pH 7.2 and 25°. Aliquots were removed for assay after varying time intervals and the pseudo-first-order rate constants of inactivation (k_I) were plotted against concentration of TNBS. Inset: double-reciprocal plot of k_I *vs.* [TNBS].

the manganese isocitrate chelate, and DPN+ had little (if any) effect.

Effect of the Concentration of Trinitrobenzenesulfonate. As shown in Figure 1, with constant enzyme concentration the increasing pseudo-first-order rate constants (k_{app}) of enzyme inactivation were not linear with trinitrobenzenesulfonate (TNBS) concentration. This suggests formation from enzyme (E) and trinitrobenzenesulfonate of a dissociable complex (E·TNBS) which is converted subsequently to the covalently linked derivative E–TNP by the sequence



$$E + TNBS \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\cdot TNBS \xrightarrow{k_2} E\text{–}TNP + HSO_3^- \quad (1)$$

By analogy with the calculations of Kitz and Wilson (1962) this can be expressed as

$$k_{app} = k_2[TNBS]/K_{TNBS} + [TNBS] \quad (2)$$

where K_{TNBS} is the apparent half-saturation constant or, when k_2 is rate limiting, $K_{TNBS} = k_{-1}/k_{+1}$. Equation 2 has the form of the Michaelis–Menten equation and a double-reciprocal plot of the rate of inactivation against TNBS concentration gives a straight line (Figure 1, inset) where the values of k_2 (0.143 min^{-1}) and K_{TNBS} (0.6 mM) could be calculated from the intercepts at the ordinate and abcissa, respectively.

Furthermore, Figure 3A shows that inactivation and trinitrophenylation of the enzyme are parallel during the initial

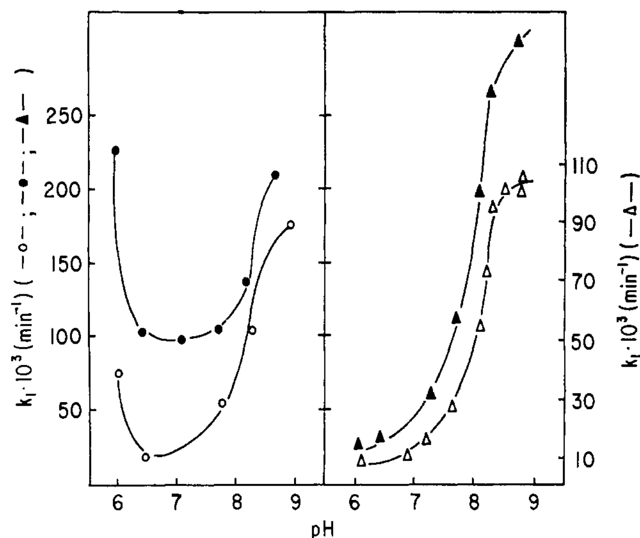


FIGURE 2: Dependence of inactivation on pH. Enzyme (0.008 mM) was incubated with 10 mM *N*-ethylacetimidate (O) or 10 mM *N*-ethylbenzylimidate (●) in mixed buffers containing 0.05 M NaHepes and 0.05 M sodium borate, or with 0.25 mM TNBS in 0.05 M NaHepes buffers in the absence (▲) or presence of 0.6 M NaCl (Δ). Pseudo-first-order rate constants of inactivation (k_I) were plotted as a function of pH. The inflection points of the curve were calculated by the best-fit polynomial method.

TABLE I: Protection against Inhibition by Various Amino Group Specific Reagents.[a]

Protector	Concn (mM)	Inhibition Rate ($k_I \times 10^3$ min^{-1}): Ethyl Acetimidate[c] (10 mM)	Ethyl Benzylimidate[c] (10 mM)	KNCO[d] (50 mM)	TNBS[e] (0.1 mM)
DL-Isocitrate	20	46.6	721	20.5	22.7
Manganese isocitrate^{-}[b]	1.9	1.12	7.42	0	0.45
Mn^{2+}	2	28.6	26.1	17.8	5.03
ADP	0.25	48.8	536	21.5	27.5
DPNH	0.2	44.6	549	26.5	7.45
DPN+	5	44.6	725	26.5	19.3
DPN+	0.5	43.1	871	20.5	19.3
None		44.3	870	25.6	26.8

[a] The enzyme, protectors and inhibitors were preincubated under the conditions described under Methods. Aliquots were removed and assayed at appropriate time intervals. [b] The concentrations of total manganese and DL-isocitrate were 2 and 20 mM, respectively. [c] The concentration of enzyme subunit was 0.0065 mM, and preincubation was in 0.05 M Na-Hepes and 0.05 M sodium borate at pH 7.2 and 25°. [d] The concentration of enzyme subunit was 0.0065 mM and preincubation was in 0.5 M NaHepes at pH 7.2 and 25°. [e] The concentration of enzyme subunit was 0.008 mM and preincubation was in 5 mM sodium phosphate buffer containing 20% glycerol at pH 7.2 and 25°.

time periods; with further incubation, inactivation slows considerably more than trinitrophenylation. A first-order plot of the enzyme inactivation data shows a linear relationship with time only until 70–80% of the enzyme activity has been lost

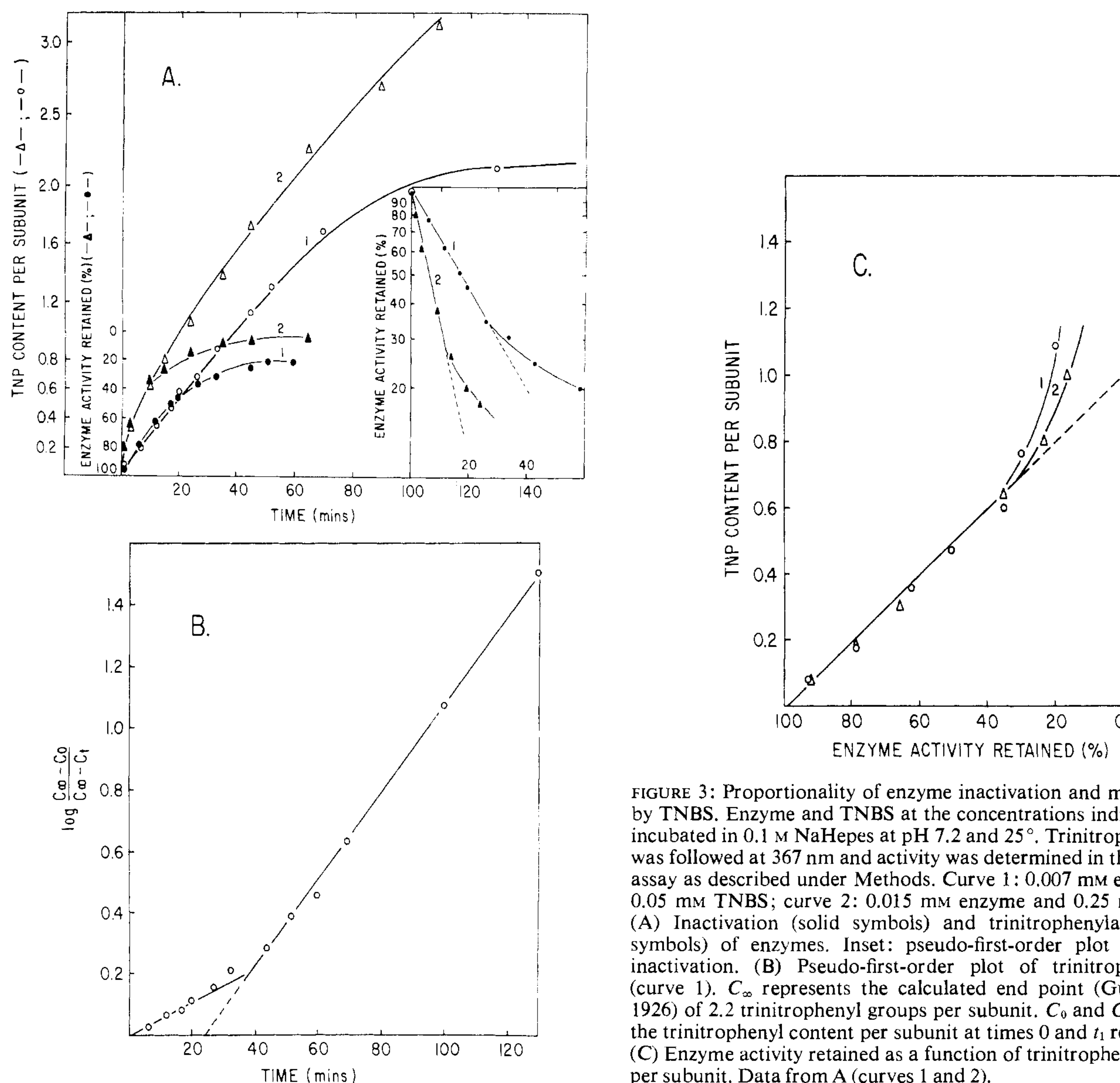


FIGURE 3: Proportionality of enzyme inactivation and modification by TNBS. Enzyme and TNBS at the concentrations indicated were incubated in 0.1 M NaHepes at pH 7.2 and 25°. Trinitrophenylation was followed at 367 nm and activity was determined in the standard assay as described under Methods. Curve 1: 0.007 mM enzyme and 0.05 mM TNBS; curve 2: 0.015 mM enzyme and 0.25 mM TNBS. (A) Inactivation (solid symbols) and trinitrophenylation (open symbols) of enzymes. Inset: pseudo-first-order plot of enzyme inactivation. (B) Pseudo-first-order plot of trinitrophenylation (curve 1). C_∞ represents the calculated end point (Guggenheim, 1926) of 2.2 trinitrophenyl groups per subunit. C_0 and C_t represent the trinitrophenyl content per subunit at times 0 and t_1 respectively. (C) Enzyme activity retained as a function of trinitrophenyl content per subunit. Data from A (curves 1 and 2).

(Figure 3A, inset). As shown in the experiment where the ratio of [TNBS]:[subunit] was 7.1 (Figure 3A, curve 1), trinitrophenylation of the protein approached a plateau after 70-min incubation. It could be calculated by the method of Guggenheim (1926) that the theoretical end point of chemical modification occurred at 2.2 groups trinitrophenylated per subunit of enzyme. A pseudo-first-order plot based on this end point indicates a biphasic curve (Figure 3B). Extrapolation of the line obtained for the later time phase of incubation shows an intercept at 23 min; this time corresponds to the point where 70% of the activity had been lost (Figure 3A, curve 1) and where the pseudo-first-order rate of enzyme inactivation began to deviate from linearity (Figure 3A, inset curve 1). A plot of the data from Figure 3A as a function of enzyme activity retained against the number of groups modified per enzyme subunit yields a straight line with a slope of 1 in the same region where enzyme inactivation follows pseudo-first-order kinetics (Figure 3C).

The results obtained during the initial phase of the reaction indicate that modification by trinitrobenzenesulfonate of eight reactive amino groups per molecule of enzyme oligomer of mol wt 320,000 leads to complete loss of activity. Since the enzyme oligomer contains eight apparently identical polypeptide chains, each with a 42,000 molecular weight (Giorgio *et al.*, 1971), the results are in accord with a stoichiometry of one rapidly reacting amino group per subunit.

Heterogeneity of Amino Groups. Heterogeneity of reactivity of amino acid residues with group specific reagents has been observed with a number of proteins (Goldfarb, 1966). When the isocitrate dehydrogenase was treated with a large excess of trinitrobenzenesulfonate under denaturing conditions, *i.e.*, with a [TNBS]:[subunit] ratio of 1000 at 95° in the presence of 1% sodium dodecyl sulfate at pH 7.2, 23.4 amino groups were trinitrophenylated per subunit in 20 min. The extent of reaction was not increased by prolonged incubation and the number of groups modified is in good agreement with 24 lysyl residues/subunit determined in a preliminary amino acid analysis.[4] Under milder conditions (2.5 mM sodium phosphate containing 10% glycerol at pH 7.2 at 25° with [TNBS]: [subunit] = 50 to 250) with the identical concentration of enzyme only 1.1 to 1.6 amino groups were modified by the sulfonate between 20 and 60 min. However, even under the latter conditions complete modification of all amino groups

[4] N. A. Giorgio, Jr., A. Yip, J. Fleming, and G. W. E. Plaut, unpublished observations (1970).

will occur upon prolonged incubation with an excess of the sulfonate (Figure 4).

A more detailed analysis of the results indicates at least four classes of trinitrobenzenesulfonate-reactive residues in the enzyme. Thus, in the presence of a limiting concentration of the sulfonate ([TNBS]:[subunit] = 7.1), one residue was modified during the first stage of the reaction leading to a loss in enzyme activity followed by trinitrophenylation of an additional group at a somewhat slower rate (Figure 3A–C, curve 1). With a larger excess of the sulfonate, modification of an additional eight to nine residues occurred (Figure 4, curves 1 and 2). The remaining 13 to 15 residues were trinitrophenylated when a large excess of reagent (2.55 mM trinitrobenzenesulfonate per 0.007 mM subunit) was used (Figure 4, curve 3); however, this final phase occurred only after a lag period suggesting that a conformational change leading to exposure of additional reactive amino groups may occur at this stage.

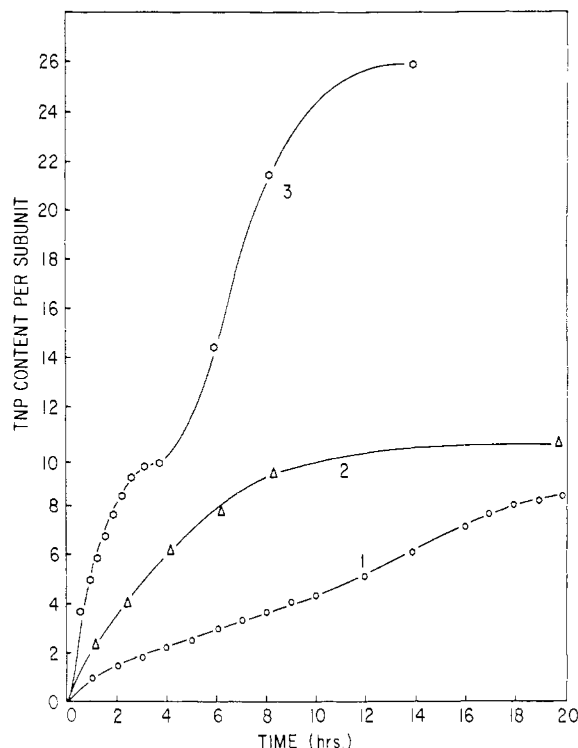


FIGURE 4: Heterogeneity of the amino groups of the enzyme. The rate of trinitrophenylation was followed in a Cary 14 spectrophotometer by the increase in absorbance at 367 nm. Enzyme and TNBS at the concentrations indicated were incubated in 0.1 M NaHepes at pH 7.2 and 25°. (○) Enzyme (0.015 mM) and TNBS (2.55 mM); (△) enzyme (0.015 mM) and TNBS (0.25 mM); (○) enzyme (0.006 mM) and TNBS (0.10 mM).

Other Reagents

Carbamylation. Treatment of the enzyme with 0.05–0.5 M potassium cyanate at pH 7.2 caused severe inhibition. The extent of modification and inactivation with time was studied with potassium [^{14}C]cyanate. When enzyme (0.023 mM) was incubated with 0.033 M [^{14}C]cyanate for two time periods, a 48 and 77% loss in activity resulted with an incorporation of radioactivity into the protein corresponding to modification of 0.56 and 0.79 residues per enzyme subunit, respectively. This suggests that carbamylation of one reactive group per subunit causes a complete loss of activity.

A combination of manganese and isocitrate protected the enzyme against inhibition by KNCO at pH 7.2, whereas isocitrate, Mn^{2+} or DPN^+ when added singly had no, or only a minor, protective effect (Table I).

Acetylation. A 95% loss of activity occurred upon incubation of the enzyme for 15 min at 23° with 2 mM *N*-acetylimidazole in imidazole buffer at pH 7.2. The modification was not accompanied by a change of absorption at 278 nm and the sulfhydryl content of native and partially inactivated enzyme were identical as measured by the method of Ellman (1959).[3] Furthermore, treatment of the partially inactivated enzyme with 0.5 M hydroxylamine at pH 7.9 and 25° for 40 min did not restore the activity. Therefore, O-acetylation of tyrosine and S-acetylation of cysteine residues probably did not occur under these experimental conditions. It is likely that acetylation of amino groups was the cause of the inactivation.

Amidination. The substituted imidoesters have been reported to react rapidly in aqueous solution at neutral pH with typical α- and ϵ-amino groups of peptides to form amidines (Ludwig and Hunter, 1967).

As shown in Table I, ethyl acetimidate and ethyl benzylimidate inhibit isocitrate dehydrogenase suggesting that the modification of an amino group(s) may be involved in this inactivation process. The rate of inactivation by ethyl benzylimidate was 20 times faster than with ethyl acetimidate and may reflect a specific interaction of the benzyl group at or near the active center of isocitrate dehydrogenase.

Striking protection agsinst inactivation by both amidinating agents (Table I) is given by manganese isocitrate and, to a lesser degree, by Mn^{2+} alone. However, when added singly, isocitrate, ADP, DPN^+, and DPNH were ineffective as protectors.

The rate of enzyme inactivation by amidinating agents is markedly dependent on pH and the profiles of pH dependency are very similar for ethyl acetimidate and ethyl benzylimidate (Figure 2). With both reagents the inflection point is above pH 8. The pronounced inactivation below pH 6.5 may not be due to amidination since studies with model compounds (Hunter and Ludwig, 1962) and with proteins (Hunter and Ludwig, 1962; Wofsy and Singer, 1963) suggest that in aqueous solution modification of amino groups occurs only between pH 7 and 10. The high rates of enzyme inactivation at pH 6 may reflect interaction of the reagents with a cation binding site which affects enzyme activity.

Discussion

The results show that of the approximately 24 lysyl residues of each subunit of isocitrate dehydrogenase one group reacts preferentially with a number of amino group specific reagents leading to loss of enzyme activity. This is particularly apparent at low concentrations of trinitrobenzenesulfonate where there is direct proportionality between loss of enzyme activity and trinitrophenylation of a single group per subunit up to 70–80% inactivation (Figure 3C). The linear pseudo-first-order rates of enzyme inactivation (Figure 3A, inset) and trinitrophenylation (Figure 3B) prevailing up to 70–80% inactivation of enzyme activity indicate that all of the reacting groups up to this point are identical. The divergence of trinitrophenylation and enzyme inactivation beyond this stage (Figure 3A) appears to be due to modification of a second group on the enzyme at a somewhat slower rate. The biphasic nature of trinitrophenylation is shown in the pseudo-first-order plots in Figure 3B. It may be that subsequent to trinitrophenylation

of the first group there is an increased reactivity of the second group, perhaps due to a time-dependent conformational change of the modified protein.

The chemical nature of the second reactive group on the enzyme is uncertain. However, it may be pertinent that while the sulfhydryl content of a preparation inactivated 50% with trinitrobenzenesulfonate was indistinguishable from that of native enzyme,[3] sulfhydryl determination by the method of Ellman (1959) of a preparation containing 2.2 trinitrophenyl groups per subunit showed a loss of 1.3 sulfhydryl group per subunit. This suggests that the second group reacting with the sulfonate may be a sulfhydryl residue.

Proportionality between inactivation and modification of a single group per subunit of enzyme was also obtained with KNCO. This is in accord with the stoichiometry of inactivation and of trinitrophenylation (Figure 3C). However, it differs from the preliminary observation of Shen and Colman (1973) who reported that with pig heart DPN-linked isocitrate dehydrogenase inactivation is proportional to carbamylation of two amino groups per subunit. The divergent results may be attributable to structural features of the enzyme from different species; however, they may also be due to the different conditions used for the modification reactions, *e.g.*, the carbamylation reactions were carried out at pH 7.2 here and at pH 7.5 by Shen and Colman (1973).

Although reaction with trinitrobenzenesulfonate caused a loss in enzyme activity proportional to trinitrophenylation the possibility still exists that this inactivation may have been due to modification of groups other than amino groups or to steric, electrostatic, or conformational effects. However, stable derivatives with groups other than sulfhydryl and amino groups have not been reported (Okuyama and Satake, 1960; Satake *et al.*, 1960). Furthermore, DPN-linked isocitrate dehydrogenase is inhibited effectively by ethyl acetimidate and ethyl benzylimidate (Table I). These compounds have been reported to react rapidly at neutral pH with free amino groups of peptides, but not with model compounds containing sulfhydryl, imidazole, or phenolic groups (Ludwig and Hunter, 1967). Since the amidine function has a pK_a near 12 the net charge of the derivatized peptide is essentially unchanged over a wide range of pH, and it has been shown that the amino groups of a number of proteins can be amidinated extensively without drastically changing the physical properties, conformation, and certain biological activities of proteins (Hunter and Ludwig, 1962; Wofsy and Singer, 1963; Pocker *et al.*, 1973).

The pH–rate profiles of enzyme inactivation by amidination and trinitrophenylation show an inflection point near pH 8 (Figure 2). This value differs by at least 2 pH units from the pK_a of the reagents (imido esters or trinitrobenzenesulfonate) or products (amidines or trinitrophenylamino groups) and, therefore, is likely to represent the pK_a of the reactive group on the protein. The fact that this pK_a is lower than that expected for a lysyl residue is in accord with a similar lowering of pK_a of special amino groups which has been observed in a number of proteins. Thus, lysine-41 in ribonuclease has a pK_a of 7 (Murdock *et al.*, 1966), the amino groups at the active site of acetoacetate decarboxylase has a pK_a of 5.9 (Schmidt and Westheimer, 1971), and Piszkiewicz *et al.*, (1971) have reported an active lysyl residue with a pK_a of 7.9 in glutamate dehydrogenase. Evidence has been presented that it is the deprotonated form of the amino group which is modified by agents such as the imidates (Hand and Jencks, 1962). The selective modification of the essential amino group at pH 7.2 of isocitrate dehydrogenase by a number of reagents noted here may well be attributable to the relatively low pK_a of this group.

Saturation-type kinetics of enzyme inactivation were observed with trinitrobenzenesulfonate (Figure 1) and seemingly with KNCO. In the case of the sulfonate, good fits of the data could be obtained to eq 2 (Figure 1, inset) which predicts formation of a dissociable intermediate enzyme–trinitrobenzenesulfonate complex (eq 1), and the value of the apparent half-saturation constant of inhibition was K_{TNBS} = 0.6 mM. In experiments similar to those shown in Figure 1 K_{TNBS} was found not to vary between pH 6.6 and 8.8. This is in contrast to the change in the pseudo-first-order constants of inactivation with pH shown in Figure 2. These results suggest that formation of the dissociable enzyme–trinitrobenzenesulfonate complex occurs at a site with a pK_a below 6.6 whereas the group on the enzyme which participates in the subsequent covalent trinitrophenylation reaction has a pK_a of 8. The reasons for the deviation from linearity of rate of inactivation with KNCO concentration are less certain. The apparent half-saturation constant of enzyme inhibition for KNCO is approximately 0.1 M. This value is about 200-fold larger than K_{TNBS} at approximately equivalent enzyme concentrations, and this concentration of KNCO is about 15,000-fold larger than that of enzyme. Thus, the nonlinearity between KNCO concentration and rates of enzyme inactivation may not merely be due to preliminary reagent binding, but could also be attributable to the effect of ionic strength, the known formation from cyanate of cyanuric acid and cyamelide (Stark, 1967), and the lack of selectivity of modification at high cyanate concentrations.

Manganese isocitrate protects the enzyme against inhibition by all of the amino group specific reagents tested (Table I), suggesting that binding of the substrate to the catalytic center interferes with access of the reagents to the reactive amino group. However, it is not possible with these data to distinguish shielding occurring by combination of the substrate with the amino group located at the catalytic site from an interaction of manganese isocitrate at the catalytic center causing a change in reactivity of the amino group at another site on the protein. Experiments on the substrate reversal of enzyme inhibition by aromatic aldehydes (Fan and Plaut, 1974) favor the former possibility.

When added singly, Mn^{2+} or DPNH have also been observed to protect the enzyme against inhibition (Table I). However, the effects are less pronounced than with manganese isocitrate and do not occur with all of the inhibitors. This indicates that protection by these components occurs by binding either at a different group(s) or with different affinities at the same group as that protected by the manganese isocitrate complex.

The protection by free Mn^{2+} against trinitrobenzenesulfonate inhibition has been studied at several concentrations of the metal ion. The dissociation constant of the enzyme–Mn complex was estimated to be K_d = 0.38 mM by the method of Mildvan and Leigh (1964). Thus, at a concentration of 2 mM Mn^{2+} used in the experiment shown in Table I, Mn^{2+} was saturating. This also indicates that the protection by manganese isocitrate against trinitrobenzenesulfonate inhibition cannot be due to free Mn^{2+}. At the concentration of total manganese and total isocitrate used to maintain manganese isocitrate at 1.9 mM (Table I), the concentration of free Mn^{2+} (0.1 mM) present would give negligible protection.

Distinct differences have been observed in degree of protection by free Mn^{2+} against inhibition by ethyl acetimidate and ethyl benzylimidate (Table I). This may suggest that

certain structural components of the reagents contribute to reactivity with the specific amino group by binding to the protein. Mn^{2+} may interfere with the binding of these secondary groups to the protein at sites other than the catalytic center. In this connection, it may be significant that protection by the divalent metal ion is more pronounced with trinitrobenzenesulfonate and ethyl benzylimidate which contain aromatic nuclei than with ethyl acetamidate or KNCO.

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