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The Reaction of a Histidine Residue in Glutamate Dehydrogenase with Diethyl Pyrocarbonate

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1. One mol of diethyl pyrocarbonate will react with one mol of glutamate dehydrogenase polypeptide chains to form one mol of N^1-carbethoxyhistidine. Reaction is prevented by NADH. 2. The 1:1 complex has an increased specific activity (1.4–2.0-fold). 3. The reason for the activation is discussed. The results are not consistent with NADH dissociation from the enzyme–glutamate–NADH complex being rate-limiting in the steady state measured. 4. The effects of modification on the properties of the enzyme were investigated. The effects of GTP and NAD^+ on the enzyme activity are unaltered by activation. NADH binding is unaltered and there is no apparent change in the molecular weight. However, the activated enzyme can still be further activated by ADP. K_a for ADP is decreased fivefold.

There is evidence for the involvement of histidine residues in the reactions of many dehydrogenases [heart lactate dehydrogenase (Berghäuser *et al.*, 1971); glyceraldehyde 3-phosphate dehydrogenase (Bond *et al.*, 1970); 6-phosphogluconate dehydrogenase (Rippa & Pontremoli, 1968); malate dehydrogenase (Anderton, 1970); α-glycerophosphate dehydrogenase (Apitz-Castro & Suarez, 1970); octopine dehydrogenase (Huc *et al.*, 1971)].

The purpose of the present paper was to test the hypothesis that glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] contains an important histidine residue. Instead of the expected loss of enzymic activity when the enzyme was incubated with diethyl pyrocarbonate, a substantial increase in activity was found. The stoicheiometry of the reaction has been investigated and the properties of the modified enzyme are discussed in the light of suggested mechanisms of the enzyme.

Materials and Methods

Bovine liver glutamate dehydrogenase was prepared and assayed for activity and protein as previously described (Holbrook *et al.*, 1973). NAD^+, NADH, GTP, ADP, ATP and 2-oxoglutarate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Diethyl pyrocarbonate and other laboratory reagents were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

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Unless stated, enzyme activity was measured by glutamate oxidation. Glutamate dehydrogenase activity was also measured in the direction of reduction of 2-oxoglutarate by NADH, by the addition of enzyme to an assay medium containing 13.4mM-sodium 2-oxoglutarate, 53mM-NH_4Cl and 0.1mM-NADH in 0.067M-NaH_2PO_4 buffer adjusted to pH7.2 with 5M-NaOH. The rate of absorbance decrease at 25°C was measured at 340nm with a Hilger–Gilford recording spectrophotometer.

Alanine dehydrogenase activity (pyruvate reduction) was measured by the method of Kallos & Shaw (1971) or alternatively in the other direction (alanine oxidation) by the method of Tomkins *et al.* (1965).

Diethyl pyrocarbonate was diluted at least 100-fold with ice-cold 0.1M-NaH_2PO_4 buffer adjusted to pH6.0 with 5M-NaOH immediately before use. The rate of reaction with glutamate dehydrogenase was studied by incubating diethyl pyrocarbonate with enzyme (approx. 1mg/ml) in this buffer. Samples were taken at known time-intervals and assayed.

Diethyl pyrocarbonate-modified enzyme was prepared by the addition of a suitable quantity (less than twice the molar quantity of enzyme, except where stated) of freshly diluted reagent to enzyme solution in 0.1M-NaH_2PO_4 buffer adjusted to pH6.0 with 5M-NaOH at room temperature. The mixture was left for approximately 1h and then assayed to determine the change in activity. After this time very little unchanged diethyl pyrocarbonate remained since it is hydrolysed very quickly to ethanol and CO_2 (Mayer & Luthi, 1960). The enzyme was therefore used directly for further experiments.

NADH binding was studied by the spectrofluorimetric-titration method of Holbrook & Stinson (1970) in 0.1M-NaH_2PO_4 buffer adjusted to pH6.0 with 5M-NaOH. The rate of NADH binding was measured by using stopped-flow fluorimetry in the same buffer. The apparatus was built by S. J. Reynolds and has been described in outline elsewhere (Wallis & Holbrook, 1973). The relative molecular mass of glutamate dehydrogenase subunits was taken as 56100.

Results and Discussion

Stoicheiometry of the reaction

Incubation of 16μM-bovine liver glutamate dehydrogenase subunits with 0.38mM-diethyl pyrocarbonate at 18°C resulted in a very rapid activation of the enzyme activity followed by a slower inhibition phase (Fig. 1). The presence of 30mM-2-oxoglutarate in the incubation mixture decreased the rate of inhibition by approximately one-third, but had no effect on the activation rate. Fig. 1 also shows the same reaction when followed spectrophotometrically at 250nm plotted on the same time-scale. At this wavelength the appearance of N^1-carbethoxyhistidine is followed. This is the product of reaction of histidine and diethyl pyrocarbonate (Mühlrád *et al.*, 1967). Fig. 1 clearly shows an initial rapid transient phase of carbethoxyhistidine production followed by a slower phase. The reaction was analysed by the method of Frost & Pearson (1961) and was found to consist of two first-order reactions. The rate constant for the rapid reaction was calculated to be 4.4min^{-1} and for the slower reaction 0.15min^{-1}. The amplitude of the initial phase is dependent on the concentration of enzyme polypeptide chains in the incubation. By using the extinction coefficient for N^1-carbethoxyhistidine of 3.6 litre·$mmol^{-1}$·cm^{-1} (Holbrook & Ingram, 1973) it was found that 1.04mol of N^1-carbethoxyhistidine/mol of enzyme subunits was present when the first initial reaction was over. Further histidine residues react slowly, causing an inhibition of the enzyme activity if excess of diethyl pyrocarbonate is present. The activation reaction is clearly due to the rapid carbethoxylation of the first histidine residue.

Titration of enzyme with diethyl pyrocarbonate produces the result shown in Fig. 2. Enzyme activity was measured after 20min to allow reaction to go to completion. Activation was complete when the molar ratio of diethyl pyrocarbonate to enzyme subunits was 1:1. Thus 1mol of diethyl pyrocarbonate reacts with 1mol of enzyme subunits to form 1mol of N^1-carbethoxyhistidine causing an increase in catalytic activity of the enzyme. Thus there can be no reaction of other amino acids in the initial phase. That reaction is with histidine is also shown by incubation

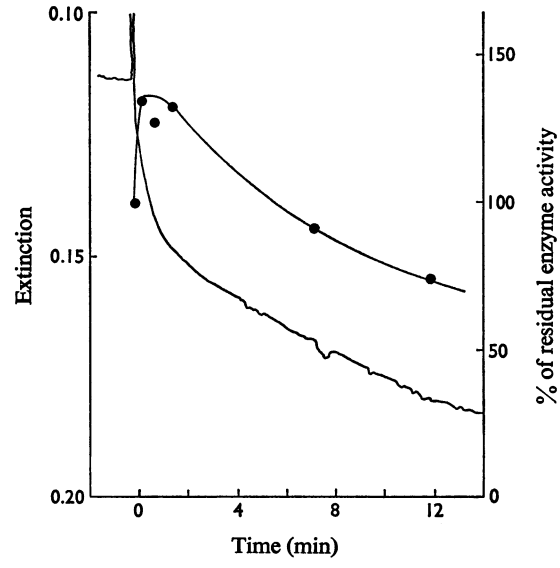

Fig. 1. *Comparison of the reaction of diethyl pyrocarbonate with glutamate dehydrogenase monitored at 250nm and by enzyme activity*

Diethyl pyrocarbonate (final concn. 0.38mM) was rapidly mixed with 16μM-enzyme subunits in a spectrophotometer cuvette. The buffer was 0.1M-NaH_2PO_4 adjusted to pH6.0 with 5M-NaOH. The absorbance change at 250nm was measured against time (solid line). An identical separate incubation was also carried out while 10μl samples were taken for measurement of enzyme activity. The relative % of residual activity of the mixture was also plotted against time (●).

of activated enzyme with 0.6M-hydroxylamine at pH7.0. This nitrogen nucleophile causes the activity to return to normal after 2.5h. The destruction of N^1-carbethoxyhistidine by hydroxylamine was originally reported by Melchior & Fahrney (1970). A control with native enzyme was unaffected by this treatment. Enzyme that had been activated by diethyl pyrocarbonate retained its high activity if diluted into buffer alone for 2.5h.

The activation of enzyme (25μM-subunits) by 0.1mM-diethyl pyrocarbonate was completely prevented if 1mM-NADH was included in the incubation mixture. Neither 1.25mM-ADP, 1mM-ATP, 0.06mM-GTP, 1mM-NAD^+ nor 30mM-2-oxoglutarate had any effect on the activation reaction.

Effect of modification on the enzyme properties

Modification by diethyl pyrocarbonate has no effect on the sedimentation coefficient of enzyme

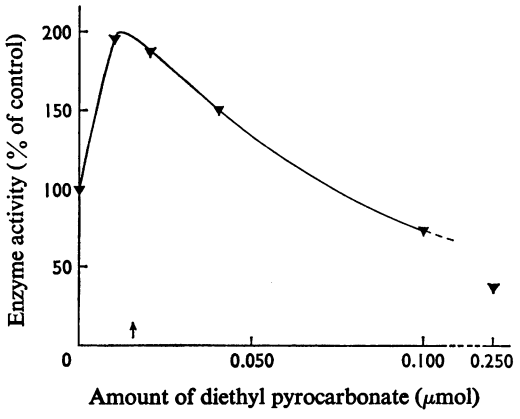

Fig. 2. *Titration of diethyl pyrocarbonate with glutamate dehydrogenase*

Samples of enzyme (16 μM-subunits) were titrated with diethyl pyrocarbonate. Each sample was left for 20 min at 25°C before enzyme activity was measured. This allowed reaction to proceed to completion. The arrow indicates the point at which the amount of reagent added was identical with the amount of enzyme subunits. The incubation medium was 1 ml of 0.1 M-sodium phosphate buffer adjusted to pH 6.0 with 5 M-NaOH.

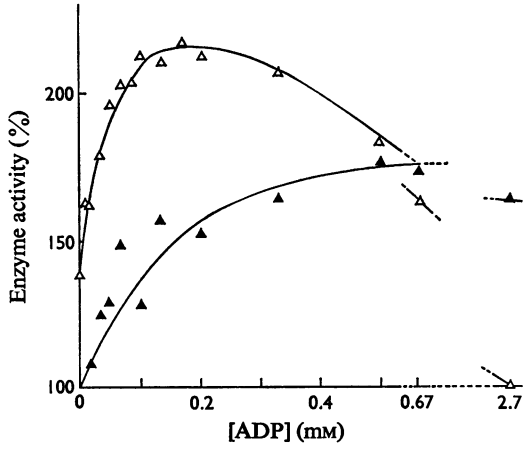

Fig. 3. *Effect of modification by diethyl pyrocarbonate on activation by ADP*

Native enzyme (▲) was assayed in the normal way and with known concentrations of ADP in the cuvette. This result is compared with a similar experiment where enzyme activated 1.4-fold by diethyl pyrocarbonate (△) was used. Activities are expressed as % of the initial assay rate caused by the same quantity of native enzyme in the absence of ADP.

(5.6 mg/ml) when measured in 0.1 M-NaH_2PO_4 buffer adjusted to pH 6.0 with 5 M-NaOH. The native enzyme had an $s_{20,w}$ of 24.9 S and the activated enzyme, 25.9 S.

The effect of the modification on the ADP-activation reaction was investigated by assaying enzyme in the normal way except that known concentrations of ADP were included in the assay medium. Fig. 3 shows that the activation of ADP is changed by

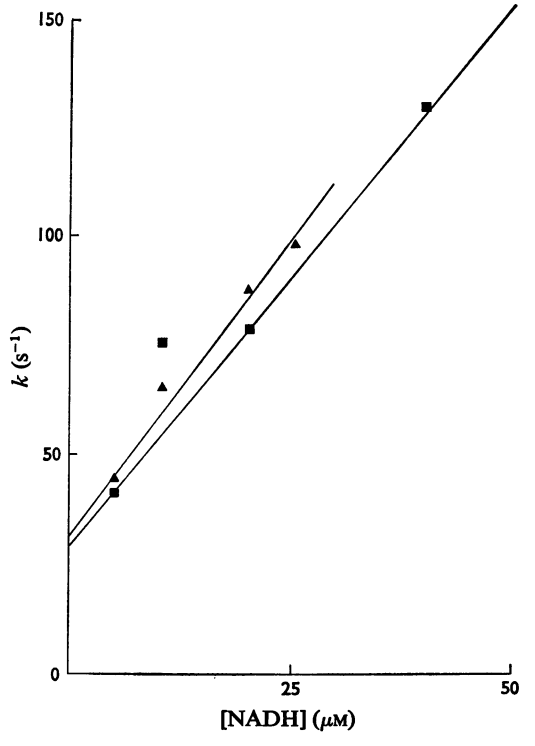

Fig. 4. *Comparison of the pseudo-first-order rate constant for NADH binding to native enzyme–glutamate complex and to the same complex with enzyme activated 200% by diethyl pyrocarbonate*

Enzyme (0.48 mg/ml) containing 8.8 mM-L-glutamate was rapidly mixed with an equal volume of buffer containing known concentrations of NADH in a stopped-flow fluorimeter. The buffer was 0.1 M-NaH_2PO_4 adjusted to pH 6.0 with 5 M-NaOH. NADH concentrations are expressed as the final concentration after mixing. The first-order rate constants (k) were calculated from oscilloscope traces of NADH fluorescence versus time at each NADH concentration. Results were obtained with native enzyme (■) and enzyme activated with diethyl pyrocarbonate (▲).

Table 1. *Effect of modification by diethyl pyrocarbonate on the catalytic reactions of glutamate dehydrogenase*

Enzyme was assayed for each activity as described in the text. Results are the activity of enzyme modified with diethyl pyrocarbonate, expressed as the percentage of the specific activity of an unmodified control enzyme. Samples A and B are considered separately.

	Activity (%)			
	A		B	
Substrate	Oxidation	Reduction	Oxidation	Reduction
Glutamate	142		170	
2-Oxoglutarate		84		
Alanine			82	
Pyruvate				175

diethyl pyrocarbonate modification. The concentration of ADP required for half-maximum activation is decreased fivefold. The diethyl pyrocarbonate-activated enzyme can still be further activated by ADP by the normal factor.

Similar experiments with different concentrations of GTP and NAD^+ showed that the effect of these nucleotides remains unchanged by modification. The maximum inhibition obtainable by raising the GTP concentration was 88% and the concentration of GTP required for one-half this inactivation was 16.7μM for both native enzyme and enzyme that had been activated 1.4-fold. The concentration of NAD^+ required for half-maximum activity was 0.71 mM for the native enzyme and 0.61 mM for enzyme activated 150% by diethyl pyrocarbonate.

Spectrofluorimetric titration with low concentrations (<0.1 mM) of NADH in sodium phosphate buffer, pH 6.0, showed that the dissociation constant and number of binding sites for NADH were unaltered after treatment with diethyl pyrocarbonate ($K_s = 18 \pm 4 \mu$M, with one binding site per enzyme subunit). This experiment was repeated in the presence of 60 mM-sodium L-glutamate. The NADH dissociation constant was considerably smaller when measured this way ($3.5 \pm 0.8 \mu$M for native enzyme). The dissociation constant of NADH in the presence of glutamate from the carbethoxylated enzyme was $1.8 \pm 0.4 \mu$M.

The effect of activation by diethyl pyrocarbonate on the other catalytic activities of the enzyme was also studied and is summarized in Table 1.

Reason for the activation

Activation of an enzyme at saturating concentrations of substrate implies that the rate of the normally rate-limiting step is increased. The work of di Franco & Iwatsubo (1971) indicates the rate-limiting step in the steady-state oxidation of glutamate by NAD^+ to be dissociation of NADH from the abortive ternary complex (enzyme–NADH–glutamate) during the first 10 s of reaction. The results in the present paper show that the NADH dissociation constant from this complex was decreased by modification of the histidine. This would tend to inhibit the reaction when measured with NAD^+ and L-glutamate as substrates and further experiments were carried out to clarify this contradiction.

The rate of NADH binding was measured in the presence of L-glutamate by stopped-flow fluorescence. This method makes use of the increased nucleotide fluorescence when NADH is bound to the enzyme (di Franco, 1971). Very little change in the second-order rate constant was observed after reaction with diethyl pyrocarbonate (Fig. 4). NADH combined with native enzyme at a rate of $2.6 \times 10^6 M^{-1} \cdot s^{-1}$ and with activated enzyme (205% of specific activity of native enzyme) at a rate of $3.0 \times 10^6 M^{-1} \cdot s^{-1}$. The NADH-dissociation rate in the presence of glutamate was calculated (from the bimolecular 'on' constant and the dissociation constant) to be $8.9 s^{-1}$ in the native enzyme and $6.0 s^{-1}$ in the activated enzyme. This would be expected to produce a 66% inhibition of the enzyme if the rate-limiting step is NADH dissociation from the enzyme–NADH–glutamate complex in the assay system used. Thus this step cannot be rate-limiting in the initial steady state used for assay purposes.

The change in apparent affinity for ADP suggested to us that the presence of ADP in the commercial NAD^+ used in the assay medium might produce an activation similar to that found. However, the concentration of ADP in the assay medium (NAD^+ and L-glutamate), assayed by the method of Adam (1963), was 0.72 μM. This is insufficient to cause any activation of the enzyme.

Thus, although carbethoxylation of the most re-

active histidine residue of glutamate dehydrogenase activates the enzyme twofold, it has not been possible to determine the individual step in the mechanism that is speeded up.

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References

Adam, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 573–578, Academic Press, New York and London

Anderton, B. H. (1970) *Eur. J. Biochem.* **15**, 562–567

Apitz-Castro, R. & Suarez, Z. (1970) *Biochim. Biophys. Acta* **198**, 176–182

Berghäuser, J., Falderbaum, I. & Woenkhaus, C. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 52–58

Bond, J. S., Francis, S. H. & Park, J. H. (1970) *J. Biol. Chem.* **245**, 1041–1053

di Franco, A. (1971) Ph.D. Thesis, Université de Paris-Sud

di Franco, A. & Iwatsubo, M. (1971) *Biochimie* **53**, 153–159

Frost, A. A. & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd edn., pp. 162–163, Wiley, New York

Holbrook, J. J. & Ingram, V. A. (1973) *Biochem. J.* **131**, 729–738

Holbrook, J. J. & Stinson, R. A. (1970) *Biochem. J.* **120**, 289–297

Holbrook, J. J., Roberts, P. A. & Wallis, R. B. (1973) *Biochem. J.* **133**, 165–171

Huc, C., Olomucki, A., Lê-Thi-Lan, Dang-Ba-Pho & van Thoai, N. (1971) *Eur. J. Biochem.* **21**, 161–169

Kallos, J. & Shaw, K. P. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 916–919

Mayer, K. & Luthi, H. (1960) *Mitt. Geb. Lebensmittel-unters. Hyg.* **51**, 132–134

Melchior, W. B., Jr. & Fahrney, D. (1970) *Biochemistry* **9**, 251–258

Mühlrád, A., Hegyi, G. & Toth, G. (1967) *Acta Biochim. Biophys.* **2**, 19–29

Rippa, M. & Pontremoli, S. (1968) *Biochemistry* **7**, 1514–1518

Tomkins, G. M., Yielding, K. L., Curran, J. F., Summers, M. R. & Bitensky, M. W. (1965) *J. Biol. Chem.* **240**, 3793–3798

Wallis, R. B. & Holbrook, J. J. (1973) *Biochem. J.* **133**, 173–182