ESSENTIAL ARGININE RESIDUES IN GLUTAMATE DEHYDROGENASE*

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1. Introduction

Positively charged functional groups of amino acid residues serve as components of recognition and binding sites in the active site of enzymes which catalyze the reaction of substrates carrying negatively charged groups as carboxylate or phosphate groups. With respect to dehydrogenases arginine residues were detected to be parts of the coenzyme binding site [2–6], and lysine or arginine residues were found to be involved in the substrate binding [2,3,6–8].

The participation of a positively charged group in the active site of glutamate dehydrogenase is suggested not only by the chemical nature of the substrates and the pyridine coenzymes but also by the fact that uncharged -SH reagents like iodoacetamide and *N*-ethylmaleimide do not alter the enzymatic activity of beef liver glutamate dehydrogenase whereas the negatively charged iodoacetic acid inactivates this enzyme irreversibly [9]. Therefore, we assume that a positively charged recognition site is essential for the orientation of the -SH reagent causing the formation of a reversible anion–protein complex before the irreversible alkylation of that cysteine residue occurs, which has been shown to be essential for the catalytic activity of glutamate dehydrogenase [9]. In addition to the functional groups necessary for the binding of the substrate and the coenzyme, positively charged functional groups are probably involved in the binding of the effectors ADP and GTP.

In this paper we describe the effect of butanedione and phenylglyoxal at pH 7.6 on the catalytic properties of glutamate dehydrogenase (EC 1.4.1.3) in an attempt to characterize the postulated recognition and binding sites. At pH 7.6 both reagents react specifically with the guanidinium group of an arginine residue [10,11].

2. Experimental

Beef liver glutamate dehydrogenase was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The protein concentration was determined spectrophotometrically at 279 nm using an $A_{1\,cm}^{1\%}$ value of 9.7 [12]. The specific activity measured with NAD^+ as the coenzyme in 0.067 M phosphate buffer, pH 7.6, as described earlier [13], was found to be 4.7 IU/mg. The amino acid analysis using short and long columns was performed according to [14]. The incubation with butanedione (5 mM) and phenylglyoxal (5 mM) was performed at room temperature in a 0.033 M phosphate–25 mM borate buffer, pH 7.6, with a protein concentration during the incubation of 1 mg/ml. After incubation the enzymatic activity was measured under standard test conditions by adding 50 μl of the incubation solution to the test mixture. Butanedione and phenylglyoxal were obtained from Fluka (Buchs, Switzerland). The nucleotides, glutamate and 2-oxoglutarate of the highest purity available were purchased from Boehringer Mannheim, buffer substances, all analytical grade, were products of E. Merck (Darmstadt, Germany). The reaction with butanedione or phenylglyoxal, respectively, must be performed in borate buffer [11].

*Studies on glutamate dehydrogenase, part XXIII; for part XXII see [1].

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3. Results and discussion

The time course for the inactivation of beef liver glutamate dehydrogenase by 5 mM butanedione and phenylglyoxal, respectively, in phosphate-borate buffer, pH 7.6, is shown in fig.1. With phenylglyoxal the reaction is almost completed after 90 min whereas with butanedione the reaction velocity is slower. After 150 min the enzymatic activity is found to be only 15–20% of that of the control. The incubation has to be performed in borate buffer with the addition of phosphate because borate without this addition inactivates the enzyme irreversibly. After 150 min e.g. an inactivation of 30% is observed in the absence compared to 0% in the presence of phosphate. In general the enzymatic activity was measured in phosphate buffer after appropriate dilution of the enzyme to the concentration needed for the determination of the enzymatic activity. However, activity measurements in phosphateborate buffer, even in the presence of 5 mM butanedione and phenylglyoxal, respectively, yield the same results as in phosphate buffer.

In order to examine if the modified guanidinium groups is involved in the interaction between the enzyme and the substrate, 2-oxoglutarate or glutamate, or the coenzyme, the modification has been performed in the presence of these compounds. No protective effect is seen by glutamate or 2-oxoglutarate. The reduced co-enzymes protect only to a small extent from the modification with phenylglyoxal (fig.1b), but to a greater extent from the modification with butanedione (fig.1a). NAD^+ has the same protective effect in the case of butanedione as well as in the case of phenylglyoxal. Combined addition of NADH and 2-oxoglutarate protects very strongly. In this case the inactivation is only 35–40% after an incubation time of 150 min. On the other hand, ADP or GTP exhibit a rather low effect with respect to the enzymatic activity during the modification reaction (fig.1).

The inactivation is reversible. After incubation during one or two hours, causing an inactivation of about 70%, the enzyme was diluted 160-fold and incubated for an additional hour. After this time the enzymatic activity was increased up to 60% compared to the control (fig.2a, e.g. for butanedione). From fig.2a it is seen that the reactivation is not a fast process. This explains why after dilution and immidiate determination of the enzymatic activity the same results were obtained in the presence and absence of borate or inhibitor (cf. above).

With both, butanedione and phenylglyoxal, the response with respect to the activating effect of ADP and the inhibiting effect of GTP is abolished (fig.2). After incubation during 150 min the residual activity is almost the same in the presence of ADP or GTP compared to the control, whereas the unmodified enzyme is activated to about 150% by ADP and inhibited to about 25% by GTP. These effects are reversed by dilution to a great extent (fig.2a).

Beef liver glutamate dehydrogenase contains 30 arginine residues per polypeptide chain. The reaction with both, butanedione and phenylglyoxal, modifies four arginine residues in the absence of protective substrates, and only two if NADH and 2-oxoglutarate are present during the reaction (table 1). Neither

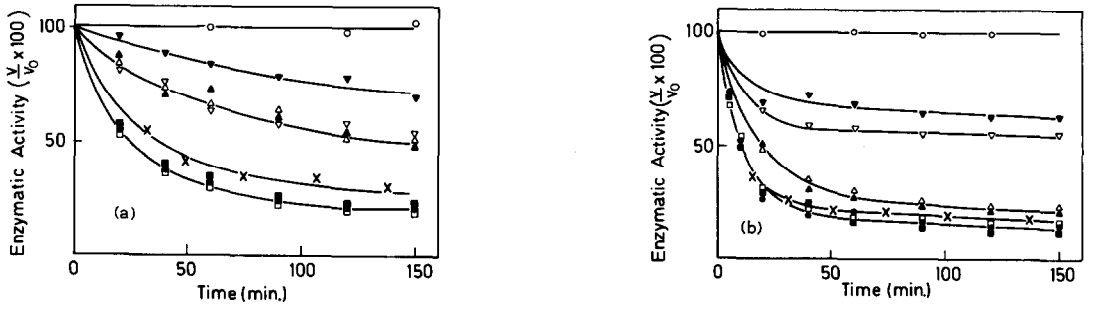

Fig.1. Time course of the enzymatic activity of beef liver glutamate dehydrogenase after incubation with butanedione (5 mM, (a)) and phenylglyoxal (5 mM, (b)), respectively. Incubation in the presence of 1 mM NADH and 11 mM 2-oxoglutarate (▼), 1 mM NADPH (▲), 1 mM NADH (△), 1 mM NAD^+ (▽), 11 mM glutamate (■), 11 mM 2-oxoglutarate (□), 300 μM ADP and 50 μM GTP (x) or in the absence of substrate (●), (○) is the control. v_0 is the initial rate of the control, v that of the modified enzyme.

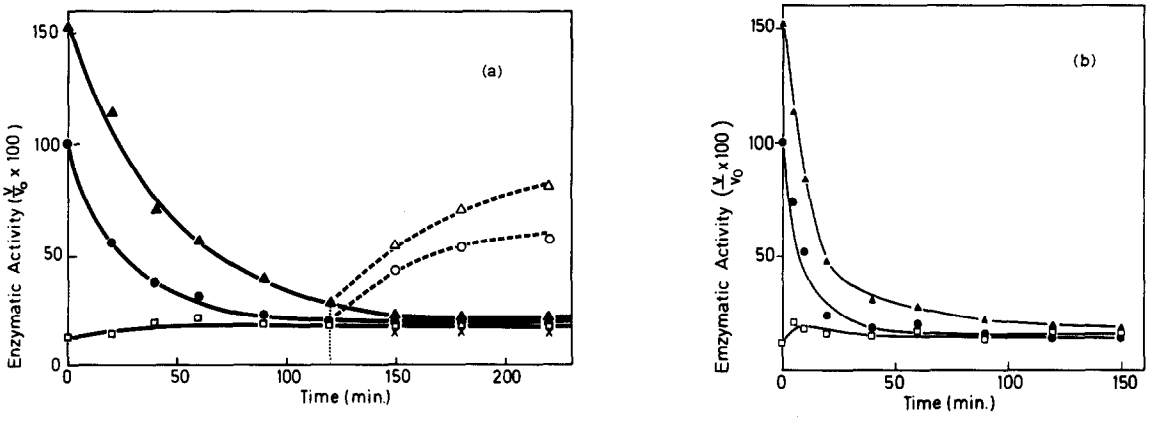

Fig.2. Effect of incubation with butanedione (5 mM, (a)) and phenylglyoxal (5 mM, (b)) on the regulatory properties of beef liver glutamate dehydrogenase. Determination of the enzymatic activity in the presence of 300 μM ADP (▲) or 50 μM GTP (□) after incubation during the indicated time in the absence of ADP and GTP, respectively. (●) is the control. The effect of dialysis was measured after incubation during 120 min for ADP (△), GTP (x), and the control (○) for the effects caused by butanedione (a).

Table 1
Protection of glutamate dehydrogenase against modification by butanedione and phenylglyoxal

Addition	Enzymatic activity[a] $(v/v_0) \times 100$		Number of Arg residues modified per polypeptide chain[b]	
	Butanedione	Phenylglyoxal	Butanedione	Phenylglyoxal
Control	22	16	4	4
Glutamate or 2-oxoglutarate, respectively (10 mM each)	22	16	3.6	3.8
NAD(P)H (1 mM)	53	25	2.8	3.8
NAD^+ (1 mM)	55	55	2.1	2.0
NADH (1 mM) and 2-oxoglutarate (11 mM)	72	63	2.0	2.0
ADP (300 μM)	27	–	2.6	2.8
GTP (50 μM)	25	–	3.0	–
(500 μM)	–	–	3.2	2.8

[a]Conditions as in fig.1, incubation time 90 min (phenylglyoxal) or 120 min (butanedione), respectively.

[b]Incubation of 1 mg/ml glutamate dehydrogenase with butanedione (5 mM, 120 min) or phenylglyoxal (5 mM, 90 min) in phosphateborate buffer, pH 7.6. Standard deviation of all experiments not exceeding 0.6 residues per polypeptide chain.

lysine, histidine nor any other amino acid residue is modified. If the modification is performed in the presence of 300 μM ADP or 50 μM GTP, respectively, one arginine residue is protected from the modification in each case.

The sedimentation analysis of butanedione-modified glutamate dehydrogenase demonstrates that the association of the unimer to higher associated particles is reduced but that the association of the six polypeptide chains within the unimer is unaffected by the modification.

Under the applied conditions both butanedione and phenylglyoxal modify only arginine residues of glutamate dehydrogenase. They display almost similar effects on the enzymatic activity, also with respect to the effectors ADP and GTP. The inhibition is accompanied by a modification of arginine residues. Whereas in the absence of protecting agents four arginine residues are modified, the coenzyme protects two residues. This figure is found too, when the substrate is present in addition to the coenzyme. Since the substrate does not have any protective effect, it can be concluded that one or two arginine residues are involved in the coenzyme but not in the substrate binding. We assume that at least one of these arginine residues is located in the active site near to the essential -SH group because the alkylation of this -SH group occurs only with negatively charged iodoacetic acid but not with uncharged -SH group reagents. Therefore, an arginine residue may act as a recognition site for the -SH group reagent and is responsible for the formation of an anion–protein complex before the alkylation takes place. Such a complex has also been postulated for the two-stage inactivation of glutamate dehydrogenase by pyridoxal 5′-phosphate, in which initial rapid, reversible binding is followed by much slower covalent-bond formation; pyridoxal, which lacks the phosphate, inactivates the enzyme without formation of a kinetically detectable non-covalent complex [15].

The fact that the activating effect of ADP and the inhibiting effect of GTP are reduced and that ADP and GTP protect one arginine residue from the modification suggests that arginine is also involved in the effector binding. The modification of three arginine residues only in the presence of ADP or GTP (table 1) compared to four in the control leaves the loss of enzymatic activity unaffected to a great extent. This indicates that the arginine residue protected by ADP or GTP is not involved in the catalytic mechanism. Since glutamate dehydrogenase has at least three different kinds of nucleotide binding site (active site, non-active site or ADP site, and GTP site) [17] it can be assumed that ADP and GTP protect different arginine residues which are also different from those protected by the coenzyme. This facilitates characterization of the different binding site by sequence analysis after modification under different protecting conditions. In this respect it is interesting to note that according to the amino acid analysis (table 1) the protecting effect of GTP is the same at low (50 μM) and high GTP concentrations (500 μM) although the dissociation constant of the enzyme–GTP complex in phosphate buffer is about 300 μM [16]. This indicates that borate increases the affinity of the enzyme for GTP.

Together with the results obtained with alcohol [4,6], lactate [2,3], and malate dehydrogenase [5] the present paper demonstrates once again that arginine is critical for the coenzyme binding and, therefore, part of the active site. From this it may be suggested that the participation of arginine residues in the coenzyme binding is a general feature in the coenzyme binding domain of pyridine nucleotide-dependent dehydrogenases. The positively charged guanidinium group of functional arginine residues is a candidate for the interaction with the negatively charged phosphate group of NAD or NADP. Since arginine residues are not only critical for the binding of coenzyme to dehydrogenases but also for the binding of ADP and GTP to glutamate dehydrogenase and for ATP to ATP requiring enzymes [18,19] to guanidinium group might be a general feature in nucleotide binding.

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