Inactivation of skeletal-muscle UDP-glucose pyrophosphorylase by reaction with carboxylate-directed reagents

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Skeletal-muscle UDP-glucose pyrophosphorylase is inactivated by reaction with 2-ethoxy-*N*-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ) and 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDAC), two reagents specific for carboxylate groups. The former reagent is a more effective inactivator than EDAC. Although no evidence of reversible enzyme–reagent complexes of the affinity-labelling type was obtained by kinetic analysis of the inactivation, the selective protection of UDP-glucose pyrophosphorylase activity against inactivation by EEDQ in the presence of uridine substrates is indicative of an active-site-directed effect. The results are consistent with the hypothesis that EEDQ modifies a single carboxylate group located in a hydrophobic domain close to the substrate-binding site, leading to enzyme inactivation. In contrast, the reaction between UDP-glucose pyrophosphorylase and EDAC appears to involve a different region of the enzyme.

INTRODUCTION

The activated glucose derivative UDP-glucose is the building block for the synthesis of glycogen, cellulose and possibly starch, and is also involved in the reactions of collagen glycosylation, in galactose metabolism and in detoxification processes [1–3]. It is synthesized from UTP and glucose 1-phosphate in the reaction catalysed by UDP-glucose pyrophosphorylase:

Much information on this enzyme has been derived from studies on UDP-glucose pyrophosphorylase from calf liver (reviewed in ref. [4]), a protein composed of eight identical subunits, each bearing a catalytic site. Binding and kinetic experiments [5–7] suggested a Bi Bi sequential kinetic mechanism; the stereochemical course of the reaction has been mapped by n.m.r. studies [8]. Relatively similar results were obtained by our group during a study of the enzyme from rabbit skeletal muscle [9,10]. In contrast with this reasonable understanding of the kinetic mechanism, details concerning the catalytic action of UDP-glucose pyrophosphorylase, including the structure of the active site, are lacking. Beside its intrinsic value, this information would be useful for the development of enzyme-targeted drugs, as suggested for the control of helminthic infestations, in particular for *Echinococcus multilocularis* [11].

These considerations led us to investigate the structure of the active site of skeletal-muscle UDP-glucose pyrophosphorylase by the classical techniques of protein modification. In the present paper we describe results obtained with 2-ethoxy-*N*-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ) and 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDAC). These reagents display a considerable selectivity for carboxylate groups, forming addition intermediates that are possibly stabilized by

nucleophilic attack by neighbouring proteins groups with eventual intramolecular cross-linking. Our results demonstrate that EEDQ is a powerful inhibitor of the enzyme via a mechanism involving the active site, since the activity is largely retained when incubations are carried out in the presence of uridine nucleotide substrates.

EXPERIMENTAL

Coupling enzymes for activity assay and substrates were purchased either from Boehringer (Mannheim, West Germany) or from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Reagent-grade EDAC and EEDQ were from Sigma Chemical Co. All chemicals employed were of analytical grade. Methods for purification of UDP-glucose pyrophosphorylase, determination of activity by a pyrophosphorolytic assay and spectrophotometric measurement of enzyme concentration were described previously [9].

For inactivation studies, the enzyme (10–30 µg/ml in 50 mM-Tris/HCl buffer, pH 7.5) was incubated for increasing periods of time at room temperature in the presence of EEDQ or EDAC added from freshly prepared stock solutions in ethanol, care being taken to maintain the final solvent concentration lower than 2% (v/v). The control incubations received an equivalent amount of ethanol. At intervals, portions of the incubation mixture were withdrawn and the activity remaining was immediately measured with a 100-fold dilution in the assay mixture. Modifications to this standard procedure are detailed in the Figure legends. During the study of the effects of pH on the inactivation by EEDQ and EDAC, it was verified that the control enzyme was stable over the whole range of pH investigated (6.0 to 9.0). The numbers of ionizable groups involved in the inactivation process at various pH values, and their pK values, were

Abbreviations used: EEDQ, 2-ethoxy-*N*-(ethoxycarbonyl)-1,2-dihydroquinoline; EDAC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide dihydrochloride.

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computed by a newly developed graphical procedure, which is described in detail in the Appendix. A limited stoichiometry for the inactivation was determined by a kinetic method [12].

Polyacrylamide-gel electrophoresis was performed on 10% slab gels by the procedure of Laemmli [13] after the samples had been boiled in the presence and in the absence of 5% (v/v) 2-mercaptoethanol. Peptide mapping after digestion with staphylococcal V8 proteinase was performed by an electrophoretic method [14].

RESULTS

EEDQ is an effective inactivator of skeletal-muscle UDP-glucose pyrophosphorylase, leading to progressive loss of activity even at concentrations as low as 25 μM at pH 7.5. The inactivation (Fig. 1) follows apparent first-order kinetics, increasing progressively as the reagent concentration is raised up to 0.5 mM; at higher concentrations of EEDQ the rate of inactivation is too high to be determined reliably. The replot of the reciprocals of EEDQ concentration against the apparent inactivation rate constants yields a straight line crossing the origin, excluding the formation of reversible enzyme-reagent complexes before inactivation takes place (Fig. 2a). Analysis of these data according to the procedure of Levy *et al.* [12] provided an approximate reaction stoichiometry of one reagent molecule per active site, since the plot of $\log[\text{EEDQ}]$ versus $\log k$ gives a line of slope 0.89 (Fig. 2b). In contrast, EDAC is a much weaker inactivator, since concentrations as high as 20 mM are required to obtain extents of inactivation comparable with those obtained in the presence of only 0.15 mM-EEDQ.

Further experiments were performed to explore the influence of pH and buffer composition on the rate of inactivation. The data presented in Fig. 3 demonstrate that the inactivation of UDP-glucose pyrophosphorylase by EEDQ is definitely faster at pH values lower than 7.0, suggesting that EEDQ recognizes groups of approximate pK in this range, acting prevalently on the protonated

Fig. 1. Time course of inactivation of skeletal-muscle UDP-glucose pyrophosphorylase by EEDQ

The enzyme was diluted with 50 mM-Tris/HCl buffer, pH 7.5, to a concentration of 20 $\mu\text{g}/\text{ml}$ and incubated with the following concentrations of EEDQ: 25 μM (●), 50 μM (▲), 100 μM (○), 150 μM (□), 300 μM (△) and 400 μM (▼). At the indicated times the residual activity was determined as described in the Experimental section.

forms; alternatively, changes in pH may induce major differences in the chemical reactivity of the reagent [15]. It is noteworthy that the pH-dependence for the inactivation of the enzyme by EDAC follows a different

Fig. 2. Replots of the data in Fig. 1 as (a) the reciprocal of the apparent inactivation rate constant against the reciprocal of the concentration of EEDQ and (b) according to the method of Levy *et al.* [12]

pattern, since higher rates of inactivation are observed in the alkaline rather than in the acid pH range. These data were processed further by the graphical method described in the Appendix to determine the number and the pK

Fig. 3. Influence of pH on the apparent inactivation rates for the reaction of UDP-glucose pyrophosphorylase with EEDQ and with EDAC

The enzyme was diluted in 0.1 M-Tris buffer adjusted to the required pH with acetic acid. the reaction was started by addition of EEDQ to give a final concentration of 0.1 mM ($\blacktriangle$) or of EDAC to give a concentration of 45 mM ($\circ$). The apparent inactivation rate constant was calculated by standard procedures from the slope of the semi-logarithmic plot of residual activity versus time.

Table 1. Influence of ligands on the inactivation of UDP-glucose pyrophosphorylase by EDAC and EEDQ

Inactivations were performed at room temperature in the presence of 50 mM-Tris/HCl buffer, pH 7.5, with 0.1 mM-EEDQ or 15 mM-EDAC. The ligands were added to the following concentrations: pyrophosphate, 2 mM; Mg^{2+} , 2 mM; UTP, 1 mM; UMP, 1.4 mM; glucose 1-phosphate, 2 mM.

Addition(s)	Apparent inactivation rate constant (min^{-1})	
	EEDQ	EDAC
None	0.185	0.092
Pyrophosphate	0.201	0.098
Mg^{2+}	0.192	0.088
Pyrophosphate plus Mg^{2+}	0.173	—
Glucose 1-phosphate	0.178	0.090
UDP-glucose	0.124	0.087
UTP	0.099	0.097
UTP plus Mg^{2+}	0.043	—
UMP	0.198	—

values of the groups involved in the inactivation. In these ways we calculated that at least two groups are involved in the inactivation by EEDQ, since the slope of the plot is 0.46; these groups have close pK values, with a mean pK of 6.75; in the case of the inactivation by EDAC a single ionization constant is involved (slope 0.86), with an apparent pK of 7.55. Furthermore, we found that even the composition of buffer affected the reaction with EEDQ, since the inactivation at pH 7.5 was slower in 50 mM-phosphate or β -glycerophosphate buffer than in 50 mM-Tris buffer, with k_{app} values of 0.085, 0.117 and 0.185 min^{-1} respectively with 0.1 mM-EEDQ. We extended these results to explore the effects of phosphorylated enzyme ligands and Mg^{2+} , an essential cofactor of UDP-glucose pyrophosphorylase, on the inactivation. In this way we observed that the substrate UTP and UDP-glucose effectively protect the enzyme from inactivation by EEDQ (Table 1); the effect of UTP is reinforced by Mg^{2+} , but other substrates and Mg^{2+} in any combination and UMP were ineffective. The protection by $MgUTP$ is saturable with respect to the nucleotide, with half-saturation at 65 μM (Fig. 4). The replot of the data according to the procedure of Scrutton & Utter [16] yields a straight line that does not cross the origin, indicating that both forms of the enzyme, the substrate-free and the substrate-saturated, are sensitive to inactivation by EEDQ with clearly different rates of inactivation. It may be noted that UTP does not exert protection against inactivation by EDAC (Table 1), suggesting again consistent differences in the action of these reagents on the enzyme.

Further efforts were directed to the preliminary characterization of the reaction with EEDQ. The reagent

Fig. 4. Protection by $MgUTP$ of the activity of UDP-glucose pyrophosphorylase during inactivation by EEDQ

The enzyme was incubated with the reagent (0.1 mM) in the standard conditions except for the addition of increasing concentrations of $MgUTP$. The apparent inactivation constant for inactivation was determined from plots of the logarithm of activity remaining versus time. In the inset the data are replotted according to the method in ref. [16].

Fig. 5. Limited proteolytic mapping of native and EEDQ-modified UDP-glucose pyrophosphorylase by digestion with staphylococcal V8 proteinase and electrophoretic separation

The native and the fully inactivated enzyme, prepared by treatment with 0.2 mM-EEDQ during 30 min, were isolated by standard SDS/polyacrylamide-gel electrophoresis and further processed according to the procedure given in ref. [14]. The proteolysis was obtained by cutting off current during 30 min as the proteins reached the separation gel. Migration is from top to bottom. The positions of unproteolysed UDP-glucose pyrophosphorylase standard and of the V8 proteinase employed in the digestion are marked by the asterisk and the doublet respectively. Lanes 1, 2, 3 and 4 are the proteolytic patterns of native (lanes 1 and 2) and modified (lanes 3 and 4) enzyme digested with 0.1 μ g (lanes 1 and 3) and 1 μ g (lanes 2 and 4) of proteinase.

usually modifies carboxylate residues to give mixed-anhydride derivatives, which are further modified by nucleophiles such as water or amino functions [15]. In the former case the modification is spontaneously reversible, but in the latter it is usually irreversible and is pushed forwards by the addition of external amino derivatives such as glycine ethyl esters. In the last instance protein cross-links are frequently produced in the case where the nucleophile is a protein amino group. The results that we obtained indicate that the reaction is not reversed by simple dialysis of the EEDQ-inactivated enzyme against Tris buffer, that a partial re-activation was provided by immediate dilution in buffers containing high concentrations of thiol reducing agents, and finally that the rate of inactivation is not influenced by the inclusion of glycine ethyl ester in the incubation. Upon electrophoresis of the inactive enzyme in the presence of SDS we detected only one band of apparent molecular mass 53000 Da, the same as that of the native enzyme, excluding the formation of intersubunit cross-links. By means of the electrophoretic proteolytic technique of Cleveland (Fig. 5) we could detect neither the disappearance nor the appearance of distinctive protein bands

in the proteolytic pattern with staphylococcal V8 proteinase; the modified enzyme is, however, definitely less sensitive than the native enzyme to the proteolytic degradation (Fig. 5, lanes 3 and 4).

DISCUSSION

Nucleotide-binding sites in proteins have been extensively investigated by spectroscopic, fluorimetric and n.m.r. techniques, as well as by chemical modification and affinity labelling [17–19]. In most instances cysteine and basic amino acid residues have been detected therein [19]. Membrane-bound enzymes, e.g. $\text{NAD}^+:\text{NADH}$ transhydrogenase [20], F_1 ATPase [21], 5'-nucleotidase [22] and $\text{Na}^+:\text{K}^+$ -dependent ATPase [23], also appear to contain aspartic acid and glutamic acid residues close to the nucleotide-binding region. Modification of these residues by EEDQ or carbodi-imides abolishes activity. A similar arrangement is probably preset at the active site of the catalytic subunit of cyclic AMP-dependent protein kinase [24]. The carboxylate groups in nucleotide-binding domains are presumably involved in the interaction of the protein with bivalent cations, which frequently act as enzyme cofactors in phosphotransferases [25].

According to our present results, UDP-glucose pyrophosphorylase also is sensitive to carboxylate-directed reagents; in particular, it appears that EEDQ is effective at micromolar concentrations. Although other substrates and UMP do not appear to influence the reaction of the enzyme with EEDQ, UTP and UDP-glucose specifically protect the enzyme from inactivation, in a manner that is reinforced by Mg^{2+} . The binding of MgUTP largely decreases the apparent rate of inactivation by EEDQ, but does not completely prevent the reaction at the EEDQ-sensitive site. The concentration of MgUTP (65 μM) required to obtain the half-maximal effect of the nucleotide is in reasonable agreement with the K_m (33 μM) calculated during a kinetic study [10]; unfortunately, this value cannot be compared directly with the dissociation constant for MgUTP , because binding data are not available for the muscle isoenzyme; however, binding and kinetic parameters correspond closely for the liver isoenzyme [7]. Our results therefore point to the involvement of EEDQ-sensitive groups in the binding of the β - and γ -phosphate moieties of UTP, in such a way that free pyrophosphate cannot protect the enzyme from the reaction with EEDQ. The inactivation by EDAC appears to involve different groups of the enzyme, as indicated by the lower reagent-sensitivity, the lack of protection by substrates and the opposite effects of pH. Similar conclusions were reached during a study of the modification of $\text{NAD}^+:\text{NADH}$ transhydrogenase by EDAC and EEDQ [26].

The reactions of EEDQ and EDAC with proteins are specific for carboxylate groups [27]: both reagents activate carboxylic groups to unstable intermediates (ketimines, *O*-acylurea derivatives or mixed anhydrides) that are stabilized by nucleophiles [24], eventually leading to protein cross-linking. We believe that this mechanism is operative also in the case of the reaction between UDP-glucose pyrophosphorylase and EEDQ; the relatively high pK of the EEDQ-sensitive group is not inconsistent with this hypothesis, since even higher values have been reported for carboxylate groups in enzyme active sites, particularly if they are located in hydrophobic domains

[28,29]. Furthermore, the reactivity of the EEDQ-sensitive group does not follow a single ionization, indicating that neighbouring groups influence the reaction with the amino acid residue acted upon by the reagent. The conclusion that the selective action of EEDQ involves the enzyme active site within a hydrophobic region of the protein is strengthened by the efficiency of EEDQ, a mildly hydrophobic reagent, in comparison with the water-soluble carbodi-imide derivative. Several aspects of the reaction, and in particular the reversibility, still require investigation; preliminary experiments have demonstrated a limited reversibility by direct dilution of the modified enzyme in thiol-containing buffers, but upon prolonged dialysis we were not able to document any recovery of activity. A possible explanation for this finding is that the labile intermediate produced during the reaction with EEDQ either undergoes hydrolysis with recovery of activity or is stabilized slowly by reaction with protein nucleophiles and production of zero-spacer intrasubunit cross-links as confirmed by the increased resistance of the modified enzyme towards proteolysis. The difficulties in the studies of reversibility also prevented absolute exclusion of the occurrence of rare side reactions of EEDQ with tyrosine or cysteine residues [30,31].

The effects of Mg^{2+} on the reaction deserve a final comment: the observation that the cation does not directly protect the enzyme but only reinforces the protection by UTP might be interpreted in terms of binding as an indication of an interaction with the enzyme-nucleotide complex, rather than with enzyme surface; this supports our previous proposal that the synthetic reaction, but not the pyrophosphorolysis, proceeds via a substrate-bridge complex [10].

At our knowledge, this is the first attempt to obtain insights into the structure of the active site of UDP-glucose pyrophosphorylase; it will be useful to elucidate further details of the reaction so that a full description can be attained in the future. It will also be of considerable interest to compare the properties of UDP-glucose pyrophosphorylase with those of other enzymes involved in the metabolism of UDP-glucose, such as glycogen synthetase and UDP-glucose dehydrogenase.

This research was supported by funds from the Italian National Research Council and from the Ministero della Pubblica Istruzione. We are grateful to Miss Daria Milani for secretarial assistance.

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APPENDIX

Development of a graphical method to determine the number and value of the ionization constants involved in pH-dependent chemical modification of proteins

The usefulness of chemical modification in the study of structural-functional relationships in proteins is widely appreciated [1,2]. Ligands usually affect the rate of reaction with chemicals either by stereochemical action

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or by modifying the reactivity of groups involved [3]. In this respect also the concentration of hydrogen ions must be considered. These considerations have been largely developed in previous studies, mostly in the case of a

selective reactivity, i.e. that only one protein species, either the protonated or the deprotonated one, is a selective target of the reaction [4]. In the more general case that both forms of the protein are modified, even with different rate constants the treatment of the data is more complex.

In general, if a sharp pH-dependence of the rate of modification is observed between two limiting rates, the process can be described by the following scheme:

where K_a is the overall dissociation constant of EH_n ($K_1, K_2 \dots K_n$) and k_1 and k_2 are the inactivation rate constants for the two protonic species. It might be calculated that the experimental rate constant for the inactivation, $k_{\text{exp.}}$, is

$$k_{\text{exp.}} = \frac{k_1 K_a + k_2 (\text{H}^+)^n}{K_a + (\text{H}^+)^n} \quad (1)$$

The usual plot of $k_{\text{exp.}}$ versus pH gives little information on the number of dissociation constants involved in the process. To this purpose, equation (1) can conveniently be rearranged in the form

$$\log \left(\frac{k_1 - k_2}{k_{\text{exp.}} - k_2} - 1 \right) = \text{p}K_a - n \text{pH} \quad (2)$$

The values of k_1 and k_2 are determined conveniently from the extreme regions of the pH-activity plot, where k_1 and k_2 are essentially independent of pH. Thus the plot of $\log[(k_1 - k_2)/(k_{\text{exp.}} - k_2) - 1]$ versus pH gives a straight line of slope n , which intersects the abscissa at $\text{p}K_a/n$; this is the mean value of the real $\text{p}K_a$ values, because K_a includes all the dissociation constants for each proton dissociation. In this way it is possible to calculate both the number of dissociations and the mean $\text{p}K$ for the reaction.

Equations (1) and (2) are correct only in the case $n = 1$. If $n > 1$, the approximation can be inadequate and the plot of equation (2) becomes curved.

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Received 6 February 1989/24 July 1989; accepted 28 July 1989