The denaturation of rabbit muscle phosphorylase *b* by guanidinium chloride

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(Received 9 February 1983/Accepted 1 June 1983)

The denaturation of phosphorylase *b* by guanidinium chloride (GdnHCl) was studied. The enzyme is unusually sensitive to the denaturing agent, being more than 50% inactivated after incubation for 15 min in 0.1 M-GdnHCl. Full activity can be regained on dilution of the GdnHCl to 0.01 M, provided that the initial concentration of GdnHCl is less than 0.5 M. Studies of protein fluorescence, thiol-group reactivity, circular dichroism and absorption spectroscopy indicate that phosphorylase *b* undergoes slow structural changes in the range of GdnHCl concentrations from 0.5 to 0.8 M. The enzyme retains considerable folded structure even after 15 min incubation in 1 M-GdnHCl, but is rapidly and completely unfolded in 3 M-GdnHCl.

Rabbit muscle glycogen phosphorylase (α -1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) has been extensively studied as an example of a regulatory enzyme (for reviews see Graves & Wang, 1972; Busby & Radda, 1976; Fletterick & Madsen, 1980). The enzyme in its *b* form has a requirement for AMP for activity (Cori & Cori, 1940) and is a dimer of subunit M_r 97 000 (Fletterick & Madsen, 1980). The amino acid sequence (Titani *et al.*, 1977) and X-ray-crystallographic structure (Weber *et al.*, 1978; Johnson *et al.*, 1979) of the enzyme have been determined. Phosphorylase contains a pyridoxal 5'-phosphate attached via a Schiff-base linkage to the side chain of Lys-679 (Titani *et al.*, 1977). The unusual absorption spectrum of the cofactor ($\lambda_{\text{max.}} \approx 335$ nm) appears to result from the hydrophobic environment provided by the folding of the protein, so that the 3-phenoxide group remains protonated (Helmreich & Klein, 1980). Unlike other pyridoxal-containing enzymes, phosphorylase retains substantial activity on reduction with NaBH₄ (Kent *et al.*, 1958).

Previous investigations (Shaltiel *et al.*, 1966; Hedrick *et al.*, 1969) have shown that the pyridoxal 5'-phosphate cofactor can be reversibly removed under 'deforming' conditions (imidazole/citrate buffer in the presence of a thiol compound such as L-cysteine). It was also noted that irreversible denaturation of the protein could be brought about by agents such as urea or GdnHCl. As part of a series of experiments (Price & Stevens, 1982, 1983)

in which we have investigated the denaturation and refolding of oligomeric enzymes, we have examined the effect of GdnHCl on phosphorylase *b*. Evidence from various studies suggests that phosphorylase *b* is unusually sensitive to GdnHCl and that unfolding occurs in a number of operationally distinct stages. Only at low concentrations of GdnHCl (≤ 0.5 M) is the unfolding reversible by dilution to give substantial regain of activity.

In the range of GdnHCl concentrations from 0.5 to 0.8 M, there is a marked decline in the regain of activity on dilution, and the structural changes in the enzyme are markedly time-dependent. In higher concentrations of GdnHCl, the structural changes are much less time-dependent.

Experimental

Materials

Phosphorylase *b* was obtained from Professor P. Cohen, Department of Biochemistry, University of Dundee, Dundee, U.K., as a solution in 50 mM-sodium glycerophosphate/2 mM-EDTA/15 mM-2-mercaptoethanol containing 50% (v/v) glycerol at pH 7.0 and stored at -18°C . Before use, samples of the enzyme solution were incubated with freshly prepared solutions of dithiothreitol (final concn. 10 mM) for at least 12 h before dialysis against buffer. Concentrations of phosphorylase *b* were determined spectrophotometrically by using values of 13.2 for $A_{280}^{1\%}$ (Birkett *et al.*, 1971) and 97 000 for the subunit M_r (Titani *et al.*, 1977). On SDS/polyacrylamide-gel electrophoresis, over 95% of the material stained by Coomassie Blue migrated as a single band.

Abbreviations used: GdnHCl, guanidinium chloride; Mes, 4-morpholine-ethanesulphonic acid; SDS, sodium dodecyl sulphate; Nbs., 5,5'-dithiobis-(2-nitrobenzoic acid); LDH, lactate dehydrogenase.

Subtilisin BPN' (proteinase type VII), pyridoxal 5'-phosphate, hide powder azure, 6-aminoheptanoic acid and cross-linked bovine serum albumin were obtained from Sigma Chemical Co. GdnHCl (AristaR grade) was obtained from BDH Chemicals. Urea (A.R. grade; Fisons) was recrystallized from ethanol. LDH-5 isoenzyme from rabbit muscle was obtained from Boehringer.

Methods

Phosphorylase *b* activity was assayed as described by Hedrick & Fischer (1965). Under these conditions the specific activity of the enzyme was 60 μmol of P_i produced/min per mg of enzyme. In the absence of AMP, the activity was less than 3% of that observed in the presence of AMP (1 mM). Reduction of phosphorylase *b* by NaBH_4 was performed as described by Graves *et al.* (1965).

Denaturation and refolding studies were performed at 20°C with a standard denaturation period of 15 min. No differences were observed between experiments performed in the presence of 2-mercaptoethanol (20 mM) and those performed in the absence of reducing agent.

Proteolysis of phosphorylase *b* by subtilisin BPN' was based on the procedure described by Raibaud & Goldberg (1973). Phosphorylase *b* (1.4 mg/ml) was incubated with subtilisin (40 μg /ml) for 30 min at 20°C before the reaction was stopped by addition of 2 mM-phenylmethanesulphonyl fluoride, and the samples were prepared for SDS/polyacrylamide-gel electrophoresis. (Control experiments with hide powder azure as substrate showed that 2 mM-phenylmethanesulphonyl fluoride caused over 95% inactivation of subtilisin within 5 min.) When the proteolysis was performed in GdnHCl concentrations above 0.5 M, cloudiness (due to insoluble guanidinium dodecyl sulphate) was observed on addition of SDS. However, a control experiment performed in 0.8 M-GdnHCl in the absence of subtilisin showed that this cloudiness did not affect the amount of protein stained by Coomassie Blue after electrophoresis.

Lactate dehydrogenase activity was monitored spectrophotometrically at 340 nm, with pyruvate and NADH concentrations of 0.8 mM and 0.2 mM respectively (20°C).

Fluorescence spectra were recorded at 20°C with a Perkin-Elmer MPF 3A spectrofluorimeter. C.d. spectra were recorded at 20°C in a Jobin-Yvon Dichrographe IV.

Most of the experiments reported in this paper were performed in 0.1 M-Mes/KOH buffer, pH 6.5. Some early experiments were performed in 0.1 M-sodium maleate buffer, pH 6.5. There were no differences between the results obtained with the two buffers.

Results

Inactivation of phosphorylase *b* in GdnHCl

Solutions of phosphorylase *b* (170 μg /ml) were incubated in the presence or absence of GdnHCl for 15 min at 20°C, before being assayed in identical concentrations of denaturing agent to prevent refolding during the assay period. The results of this experiment are shown in Fig. 1. It is clear that phosphorylase *b* is very sensitive to GdnHCl, with approx. 55% of activity lost in 0.1 M-GdnHCl and over 95% of activity lost in 0.3 M-GdnHCl. This sensitivity is considerably greater than that of other oligomeric enzymes, e.g. LDH-5 isoenzyme (Fig. 1), rabbit muscle creatine kinase (Bickerstaff *et al.*, 1980), rabbit muscle pyruvate kinase (Price & Stevens, 1983) and yeast phosphoglycerate mutase (N. C. Price, unpublished work) none of which is inactivated to any measurable extent by prolonged incubation in 0.1 M-GdnHCl. As also shown in Fig. 1, reduced phosphorylase *b* is inactivated similarly to the native enzyme.

Reversal of inactivation by dilution

A commonly used procedure to study the refolding of an enzyme is to dilute a solution of denatured enzyme to such an extent that the residual concentration of denaturing agent is without effect on the properties of native enzyme (see, e.g., Bickerstaff *et al.*, 1980; Jaenicke *et al.*, 1981; Price & Stevens, 1983). The results in Fig. 1 indicate that

Fig. 1. Inactivation of phosphorylase *b* and lactate dehydrogenase by incubation in GdnHCl

Phosphorylase *b* (170 μg /ml) was incubated for 15 min at 20°C in 0.1 M-Mes/KOH buffer, pH 6.5, containing the stated concentrations of GdnHCl, before assay in the same stated concentrations. ○, Native phosphorylase *b*; ●, reduced phosphorylase *b*; △, LDH-5 isoenzyme incubated under similar conditions.

for phosphorylase *b* the residual concentration of GdnHCl must be very low indeed; a value of 0.01 M was chosen. When this degree of dilution was adopted, it was found that the regain of activity after 15 min incubation in GdnHCl was markedly dependent on the concentration of denaturing agent (Fig. 2*a*). In the concentration range up to 0.5 M-GdnHCl, nearly 100% re-activation (relative to a non-denatured control) could be obtained. Above this concentration the extent of re-activation declined sharply, such that at 0.8 M-GdnHCl and above no activity was regained.

The regain of activity on dilution was rapid; the activity shown in Fig. 2(*a*) was regained after 2 min or less. There was no change in activity over a further 60 min after dilution.

If, instead of rapid dilution, the concentration of GdnHCl was lowered from 1 M to 0.01 M more slowly by a series of sequential dialyses against solutions of progressively lower concentrations of GdnHCl, there was extensive precipitation of the enzyme and no regain of activity.

Further experiments revealed that there was a time-dependence of the effects shown in Fig. 2(*a*). After 10 s incubation in 0.8 M-GdnHCl, phosphorylase *b* regained nearly 90% of activity on dilution of the GdnHCl to 0.01 M (Fig. 2*b*), whereas after 2 min incubation in 0.8 M-GdnHCl the extent of re-activation was less than 5%. At a higher concentration of GdnHCl (2 M) there was no difference in the extents of re-activation (<1%) of samples incubated for 10 s and 15 min before dilution.

The effects of the ligands AMP, glucose 6-phosphate and pyridoxal 5'-phosphate on the re-

activation of phosphorylase *b* were studied. AMP (10 mM) gave a small but definite protection, shifting the curve for native enzyme to the right (Fig. 2*a*). Glucose 6-phosphate (10 mM) gave a similar degree of protection (results not shown). Pyridoxal 5'-phosphate (0.2 mM) was without effect. Reduced phosphorylase *b* showed a similar type of curve to that of native enzyme, but shifted to lower concentrations of GdnHCl (Fig. 2*a*).

Reactivity of thiol groups

Phosphorylase *b* possesses two rapidly reacting thiol groups per subunit that can be modified by reagents such as Nbs₂ or iodoacetamide (Graves & Wang, 1972). The reaction of enzyme with Nbs₂ can be used as a measure of the structural changes brought about by GdnHCl; the results of such experiments are shown in Fig. 3. In the absence of GdnHCl, two thiol groups reacted rapidly with Nbs₂, with only a very slow subsequent reaction (approx. 0.2 group/subunit after 30 min). After 15 min incubation in 0.4 M-GdnHCl, there were still two rapidly reacting thiol groups, but the subsequent reaction was more rapid. A point of inflexion was noted in the progress curve after about 5 min reaction, and the subsequent increase in absorbance at 412 nm was accompanied by increasing turbidity of the solution. The extent of reaction after 30 min, shown in Fig. 3, was determined after centrifugation (1000 *g* for 1 min) to remove denatured protein. Eight thiol groups per subunit had reacted after 60 min. After 15 min incubation in 0.8 M-GdnHCl, the number of rapidly reacting thiol groups had increased to approx. 4 (Fig. 3); after 30 min

Fig. 2. Re-activation of phosphorylase *b* by dilution

(*a*) Enzyme was incubated for 15 min at 20°C in 0.1 M-Mes/KOH buffer, pH 6.5, containing the stated concentrations of GdnHCl. The samples were then diluted to a final GdnHCl concentration of 0.01 M and enzyme concentration 50 µg/ml and assayed. ○, Native enzyme; ●, native enzyme with 10 mM-AMP included during denaturation and re-activation; △, reduced enzyme. (*b*) Enzyme was incubated at 20°C in 0.1 M-Mes/KOH buffer, pH 6.5, containing 0.8 M-GdnHCl, for various periods of time before an 80-fold dilution and subsequent assay.

Fig. 3. Reaction of thiol groups of phosphorylase *b* with Nbs_2 .

Enzyme (0.75 mg/ml) was incubated at 20°C in 0.1 M-Mes/KOH buffer, pH 6.5, containing the stated concentrations of GdnHCl. Nbs_2 (250 μ M) was added after 15 min incubation, and the extents of reaction were determined after 15 s (O) and 30 min (●). Δ refers to samples incubated in GdnHCl for 15 s before addition of Nbs_2 , the extents of reaction being measured after 15 s reaction.

essentially all thiol groups had reacted. The limiting value of approx. 8.4 groups per subunit compares well with the total of 9 cysteine residues located in the amino acid sequence of phosphorylase (Titani *et al.*, 1977). In 3 M-GdnHCl, all thiol groups reacted rapidly with Nbs_2 (Fig. 3).

Fig. 3 also shows the number of rapidly reacting thiol groups determined after a short period (15 s) of incubation in different concentrations of GdnHCl before addition of Nbs_2 . The trend is similar to that observed after the 15 min incubation period, but it is clear that in the range of GdnHCl concentrations from 0.5 to 0.8 M there was a greater number of rapidly reacting thiol groups after the longer incubation period, presumably as the result of slow structural transitions in the enzyme.

In 1 M- or 3 M-GdnHCl little or no time-dependence of the number of reactive thiol groups was observed.

These data suggest that, at GdnHCl concentrations up to about 0.4 M, the structure of the enzyme in the proximity of the thiol groups is not greatly affected, so that only the rapidly reacting

groups [likely to be those of Cys-171 and Cys-317 (Graves & Wang, 1972; Fletterick & Madsen, 1980)] are accessible to Nbs_2 . However, when these side chains have been modified, the structure of the enzyme is sufficiently destabilized to allow progressive unfolding, reaction of other side chains, and precipitation of the protein. In the range of GdnHCl concentration from 0.5 to 0.8 M, the number of exposed (immediately reactable) thiol groups increases in a time-dependent manner to approx. 4. The extra side chains may well be those of Cys-108 and Cys-142, which are known to react very slowly in the native enzyme (Graves & Wang, 1972; Fletterick & Madsen, 1980). The finding that, in 1 M-GdnHCl, approximately half the thiol groups remain unavailable for rapid reaction with Nbs_2 indicates that the enzyme retains considerable folded structure. By this criterion, the enzyme is rapidly and completely unfolded in 3 M-GdnHCl (Fig. 3).

Proteolysis of phosphorylase *b* with subtilisin

Raibaud & Goldberg (1973) showed that subtilisin cleaves phosphorylase into two major fragments, of M_r 70000 and 30000. The bond cleaved (Gln-264-Ala-265) occurred in a loop of the polypeptide chain (Weber *et al.*, 1978; Johnson *et al.*, 1979; Fletterick & Madsen, 1980). Since subtilisin is unaffected by GdnHCl up to 1 M, it was used to examine the progressive unfolding of phosphorylase (for conditions see the Experimental section). Under these conditions SDS/polyacrylamide-gel electrophoresis shows bands at M_r 97000 and 70000, being the intact polypeptide and the C-terminal fragment, residues 265–841. From 0.4 M- to 0.5 M-GdnHCl, the fragment of M_r 70000 increases progressively; in 0.6 M-GdnHCl fragments of M_r 54000, 41000 and 25000 become more significant, and in 0.7 M-GdnHCl little of the 70000- M_r fragment remains and low- M_r material predominates. We conclude that the C-terminal two-thirds portion becomes substantially unfolded from 0.6–0.7 M-GdnHCl onwards.

Protein fluorescence

The fluorescence emission spectra of phosphorylase *b* were obtained in GdnHCl concentrations of 0, 1 M and 3 M (in each case excitation was at 290 nm). In 3 M-GdnHCl the peak fluorescence was quenched to 40% and red-shifted from 335 nm to 350 nm. In 1 M-GdnHCl the peak fluorescence was quenched to 65% and was not shifted. From 0 to 0.5 M-GdnHCl, there was relatively little change in the fluorescence intensity; between 0.5 M- and 1 M-GdnHCl a gradual quenching was observed, and the changes were time-dependent, up to 15 min; in 3 M-GdnHCl no time-dependence was observed.

Circular dichroism

As noted by Johnson & Graves (1966), phosphorylase *b* shows dichroic absorption not only in the aromatic region of the spectrum (270–290 nm) but also at 335 nm, attributable to the protein-bound pyridoxal 5'-phosphate cofactor. The c.d. spectra of phosphorylase *b* in GdnHCl concentrations of 0, 0.1 M, 0.53 M, 0.8 M and 3 M are shown in Fig. 4(a) (each spectrum was recorded 10 min after addition of GdnHCl). From these spectra the following conclusions can be drawn.

Firstly, the band at 335 nm remained largely unaffected at GdnHCl concentrations up to about 0.5 M (Figs. 4a and 4b), indicating that the environment of Lys-679 is not significantly affected

over this range of denaturant concentrations. In the range of GdnHCl concentrations from 0.5 M to 0.8 M the band at 335 nm essentially disappeared, showing that the environment of the cofactor was changed substantially.

Secondly, in the 270–290 nm region characteristic of absorption by tyrosine and tryptophan there were progressive small changes in the spectrum over the range of GdnHCl concentrations up to 0.8 M. However, even in 0.8 M-GdnHCl, the enzyme retains considerable folded structure, as revealed by the persistence of the dichroic absorbance. In 3 M-GdnHCl, the c.d. spectrum disappeared completely, indicating the loss of folded structure (Fig. 4a).

Fig. 4. Circular dichroism of phosphorylase *b*

Spectra were recorded at 20°C in 0.1 M-Mes/KOH buffer, pH 6.5. (a) Spectra recorded in the wavelength range 255–380 nm in the absence of GdnHCl (—), in the presence of 0.1 M (---), 0.53 M (.....), 0.8 M (----) and 3 M-GdnHCl (— · —) respectively. In each case the spectrum was recorded 10 min after addition of GdnHCl, and the enzyme concentration was 1.1 mg/ml (pathlength 10 mm). (b) Changes in $[\theta]_{m.r.w.}$ at 335 nm as a function of GdnHCl concentration. Conditions were as in (a). (c) Spectra recorded in the wavelength range 202–250 nm in the absence of GdnHCl (A) and in the presence of 1 M (B) and 3 M (C) GdnHCl. Spectra were recorded 10 min after addition of GdnHCl and the enzyme concentration was 1.8 mg/ml (pathlength 0.1 mm). Other conditions were as in (a). (d) Changes in $[\theta]_{m.r.w.}$ at 220 nm as a function of GdnHCl concentration, measured 1 min (○) and 15 min (●) after addition of GdnHCl. Other conditions were as in (c).

The c.d. spectra of phosphorylase *b* in the absence and presence of GdnHCl were also recorded in the wavelength range 200–250 nm, with the results shown in Fig. 4(c). In the absence of GdnHCl the spectrum was characteristic of a protein with considerable helical secondary structure, as described previously (Johnson & Graves, 1966). By using the reference value for $[\theta]_{m.r.w.}$ at 225 nm for helical proteins (Chen *et al.*, 1974), the helical content of phosphorylase *b* can be calculated as approx. 45%, in good agreement with the value (50%) obtained by X-ray crystallography (Fletterick & Madsen, 1980). The spectrum obtained in 1 M-GdnHCl shows that considerable secondary structure (approximately two-thirds that of the native enzyme) is retained under these conditions. In 3 M-GdnHCl, there was no evidence of distinct secondary structure.

Fig. 4(d) shows the results of studies in which $[\theta]_{m.r.w.}$ at 220 nm was measured as a function of GdnHCl concentration. There was little, if any, change in the secondary structure content of phosphorylase at concentrations of GdnHCl up to 0.5 M. In higher concentrations of GdnHCl (0.6 M, 0.7 M and 0.8 M), time-dependent decreases in the secondary structure were observed (Fig. 4d). In 1 M- and 3 M-GdnHCl there was no time-dependence over the 15 min period.

Absorption spectrum of phosphorylase *b*

The unusual absorption spectrum of pyridoxal 5'-phosphate bound to phosphorylase *b* (λ_{max} 335 nm) has been attributed to a Schiff base in which the 3-phenoxide group remains protonated; the λ_{max} for a normal Schiff base (i.e. with the 3-phenoxide group ionized) is 415 nm (Helmreich & Klein, 1980). Under the conditions of the present studies the λ_{max} of free pyridoxal 5'-phosphate is 388 nm. It is therefore possible to use the absorption spectrum of phosphorylase *b* to monitor conformational changes brought about by GdnHCl, as has been done for the acid- and alkali-induced unfolding of the enzyme (Kent *et al.*, 1958) and for the conformational change brought about by the reaction with iodoacetamide (Avramovic-Zikic *et al.*, 1970). The release of pyridoxal 5'-phosphate can be tested by dialysis (Kent *et al.*, 1958). Control experiments showed that (i) GdnHCl up to at least 2 M had no significant effect on the absorption spectrum of pyridoxal 5'-phosphate or on the Schiff base formed between pyridoxal 5'-phosphate and 6-aminohexanoic acid, and (ii) when 1 ml of a solution of pyridoxal 5'-phosphate (300 μ M) was dialysed against 3 ml of buffer the concentration of pyridoxal 5'-phosphate in the diffusate reached 50% of its equilibrium value in 30 min and 95% of its equilibrium value in 150 min. The rate of dialysis was

somewhat lower in the presence of GdnHCl, with the concentrations of pyridoxal 5'-phosphate reaching 93, 86 and 83% of their equilibrium values in 0.5 M-, 0.8 M- and 2 M-GdnHCl respectively after 150 min.

Solutions of phosphorylase *b* were incubated with various concentrations of GdnHCl, and the absorption spectra were recorded 10 s after addition of GdnHCl and at intervals in the next 15 min. After this period, each sample was transferred to a dialysis sac and dialysed against 3 ml of buffer containing the same concentration of GdnHCl as had been present in the original incubation mixture. The absorption spectrum of the diffusate was recorded at intervals from 30 min to 4 h after the start of dialysis. The concentration of pyridoxal 5'-phosphate in the diffusate was calculated from $\epsilon_{388} = 4900 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Gunsalus & Smith, 1957).

In 0.6 M-GdnHCl, a small absorbance at 420 nm was observed after addition of the denaturing agent, corresponding to a Schiff base in a hydrophilic environment. The absorbance at 420 nm apparently increased during the 15 min incubation period, but this was at least partly due to the increasing turbidity of the solution. The release of pyridoxal 5'-phosphate on dialysis was 0.6 mol/mol of subunit after 4 h. In 0.7 M- and 0.8 M-GdnHCl, the increases in absorbance in the 420 nm region after addition of GdnHCl were more marked, and the development of turbidity more pronounced and rapid. The release of pyridoxal 5'-phosphate were 0.9 and 1.3 mol/mol of subunit in the subsequent dialysis.

Similar results were obtained in higher concentrations of GdnHCl (2 M and 4 M), i.e. very rapid formation of a peak at approx. 420 nm and release of pyridoxal 5'-phosphate on subsequent dialysis.

The conclusion from these studies is that, in the range of GdnHCl concentrations (0.6–0.8 M) in which loss of activity becomes irreversible by dilution (Fig. 1), there is rapid unfolding of the enzyme in the region of Lys-679, exposing the pyridoxal 5'-phosphate group to a hydrophilic environment. The development of turbidity is accompanied by release of the cofactor from the enzyme, as detected by the subsequent dialysis.

Effect of urea on phosphorylase *b*

Experiments analogous to those summarized in Figs. 1 and 2(a) were performed with urea as denaturing agent (in 0.1 M-sodium maleate buffer, pH 6.5). Phosphorylase *b* was much more susceptible to urea than was LDH-5 isoenzyme; a 15 min incubation in 0.4 M-urea at 20°C resulted in a 50% loss of phosphorylase activity, but no loss of lactate dehydrogenase activity. Experiments in which phosphorylase *b* was incubated in urea solutions of different concentration for 15 min and then diluted to give a residual urea concentration of 0.1 M showed that full activity (relative to a control

sample not treated with urea) could be regained in the range of concentrations up to 2M. In concentrations above 2M, dilution led to progressively smaller extents of re-activation, so that after incubation in 3.6M-urea no re-activation was observed on dilution. As with GdnHCl, it was found that inclusion of 2-mercaptoethanol (20mM) was without effect, although AMP (10mM) afforded slight protection, analogous to that depicted in Fig. 2(a).

Discussion

Phosphorylase *b* is clearly much more sensitive to denaturation by GdnHCl than are a number of other oligomeric enzymes, such as lactate dehydrogenase (Fig. 1), creatine kinase (Price & Stevens, 1982), pyruvate kinase (Price & Stevens, 1983) and phosphoglycerate mutase (N. C. Price, unpublished work). The substantial inactivation brought about by 0.2M-GdnHCl (Fig. 1) must be the result of subtle changes in the geometry of the active site of the enzyme, since this concentration of denaturant has little measurable effect on the number of reactive thiol groups (Fig. 3), the susceptibility to subtilisin, protein fluorescence, secondary structure (Fig. 4d) or the environment of the pyridoxal 5'-phosphate cofactor.

The structural changes brought about by 15 min incubation with GdnHCl at concentrations up to 0.5M are reversible, as judged by the regain of activity on dilution to 0.01M-GdnHCl (Fig. 2a). In the range of GdnHCl concentrations from 0.5M to 0.8M, there was a sharp fall in the extent of re-activation on dilution after a 15 min incubation period (Fig. 2a). This range of concentrations also corresponds to major structural changes in the enzyme. Apart from the general changes detected by changes in the susceptibility to proteolysis, in the protein fluorescence and in the secondary structure, there is also evidence for specific changes in the environments of two cysteine side chains (possibly Cys-108 and Cys-142) (Fig. 3), and in the environment of the pyridoxal 5'-phosphate attached to Lys-679. However, several measurements (fluorescence, circular dichroism and thiol-group reactivity) indicate that even after incubation for 15 min in 0.8M-GdnHCl a considerable degree of ordered structure is still retained in the enzyme.

One aspect of the denaturation of phosphorylase *b* by GdnHCl is that many of the changes were time-dependent, especially in the range of GdnHCl concentrations from 0.6M to 0.8M. A further observation, which can represent a problem in various spectroscopic studies, was that over a period of time solutions of the enzyme became turbid. In concentrations of GdnHCl up to 0.5M, this was a slow process, with only slight turbidity evident after 4 h incubation at 20°C. In 0.8M-GdnHCl, the

development of turbidity was much more rapid and could usually be noted during a 15 min incubation period. No turbidity was observed on incubation in high concentrations of GdnHCl (3 or 4M) for 4 h. Turbidity was also noted on dilution of samples containing GdnHCl at concentrations above 0.6M to yield final concentrations below 0.6M. Slow conformational transitions have also been observed in studies of denaturation of pig muscle lactate dehydrogenase by GdnHCl (Jaenicke *et al.*, 1981) and by acid (Zettlmeissl *et al.*, 1981).

The rapid structural changes in phosphorylase *b* brought about by incubation in 0.8M-GdnHCl to expose the previously buried pyridoxal 5'-phosphate attached to Lys-679 to a hydrophilic environment do not cause the inability to regain activity on dilution (see Fig. 2b). It seems probable that this inability is instead correlated with the development of turbidity, reflecting the susceptibility of the rapidly formed, partially unfolded, structure to more extensive irreversible unfolding. The lack of time-dependence of fluorescence changes and other properties at high concentrations of GdnHCl (3M) suggests that under these conditions a highly unfolded state is formed rapidly (within 20 s).

It is not yet possible from the X-ray-crystallographic structure to determine the cause of the marked susceptibility of the enzyme to denaturation by GdnHCl (and by urea). However, it may be associated with the conformational flexibility of phosphorylase, the activity of which is known to be regulated by many different ligands (Busby & Radda, 1976).

We thank the Science and Engineering Research Council for general financial support, and Professor Roger Pain, University of Newcastle upon Tyne, for help in obtaining and interpreting c.d. spectra. Thanks are also due to Miss Jane Smyth for help with some of the initial experiments.

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