Early conformational changes and activity modulation induced by guanidinium chloride on intestinal alkaline phosphatase

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Moderate concentrations of guanidinium chloride induce both instantaneous and time-dependent modifications of the catalytic and optical properties of intestinal alkaline phosphatase, which undergoes consecutive conformational transitions at about 0.05 M, 0.25 M and 1.0 M denaturant. A paradoxical activation is observed up to 1.0 M-guanidine, with a maximum at 0.25 M- and a mid-point around 0.5 M-guanidine. Difference absorbance and fluorescence spectra imply a change in the state of ionization of the protein residues, with variation in molecular size suggested by light-scattering. Random-coil formation is indicated by a lower fluorescence yield, a more polar environment of the aromatic residues and another separate tryptophan emission. Iodide quenching confirms the alterations of conformation. Deprotonation favours the loss of the intramolecular constraints and the enhancement of the structure disruption by guanidine.

INTRODUCTION

The isoenzymes of alkaline phosphatase (AP) have been shown to display very complex patterns of inhibition when treated with moderate concentrations of urea (Miggiano *et al.*, 1984), thiourea and guanidine (Miggiano *et al.*, 1986), but detectable conformational changes have been found only with elevated concentrations of denaturants (Kadner & Nyc, 1965; Schlesinger, 1965; Fosset *et al.*, 1974).

The kinetic behaviour of the intestinal form diverges from that of other AP isoenzymes upon the addition to the reaction mixture of different modifiers such as urea (Miggiano *et al.*, 1984) and EDTA (Conyers *et al.*, 1967; Miggiano *et al.*, 1985a).

The aim of the present work is to correlate the kinetic pattern of calf intestinal AP, treated with moderate concentrations of guanidinium chloride (GndHCl), with the concurrent structural modifications.

EXPERIMENTAL

Chemicals

Crystalline GndHCl (grade I; Sigma Chemical Co., St. Louis, MO, U.S.A.) was recrystallized in accordance with Nozaki & Tanford (1967). Absorbance at 225 nm of a 6.0 M solution in water was about 0.15, with no significant absorption above 250 nm and no fluorescence at all. Tris buffer, from Merck (Darmstadt, West Germany), was in reagent-grade water obtained from a Milli Q apparatus (Millipore Corp., Bedford, MA, U.S.A.); *p*-nitrophenyl phosphate was from Carlo Erba (Milan, Italy). All other chemicals were of the best quality commercially available.

Enzyme preparation and assay

Calf intestine AP (molecular-biology grade; Boehringer, Mannheim, West Germany) was diluted in 100 mM-Tris buffer, pH 9.9. Purity was ascertained by polyacrylamide-gel electrophoresis in the presence of SDS in accordance with Weber & Osborn (1969), and only one band could be detected. Specific activity was 2000 units/mg when assayed as follows. Protein concentration was determined from absorbance measurements at 278 nm, by using $A_{1\text{ cm}}^{1\%} = 7.6$ as given by Fosset *et al.* (1974). The enzymic activity was determined as previously described (Miggiano *et al.*, 1985b) from the rate of hydrolysis of *p*-nitrophenyl phosphate (10 mM) in 100 mM-Tris buffer, pH 9.9, containing 2 mM-MgCl₂, at 37 °C, by measuring the absorbance changes at 404 nm with an HP 8450A UV-VIS spectrophotometer (Hewlett-Packard Co., Palo Alto, CA, U.S.A.). The reaction was started by the addition of the enzyme. Inhibited activity was assayed with GndHCl included in the reaction mixture. Control and inhibited activity was also determined at various pH values from 8.6 to 11.1, measured on a PHM 84 Research pH-meter (Radiometer, Copenhagen, Denmark). All experiments were carried out at least in triplicate.

Spectroscopy

The absorbance spectra were measured with the above-specified diodes-array spectrophotometer by reading the test cuvettes containing the protein (0.11 mg/ml) plus the effector, immediately after the addition of the last reactant and rapid mixing, against a blank with the same amount of GndHCl and no protein. Difference spectra were obtained by subtracting the spectrum of the untreated protein (control) from that of the treated one.

Abbreviations used: AP, alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]; GndHCl, guanidinium chloride.

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Correction for light-scattering was in accordance with Leach & Sheraga (1960).

Fluorescence titrations were performed as previously described (Martorana *et al.*, 1986) by using an LS5 Luminometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.) with software for the correction of spectra. Experimental conditions were as follows: to 450 μ l portions of reaction solution with different concentrations of GndHCl were added 50 μ l of protein (0.11 mg/ml) in the sample cuvettes and 50 μ l of the buffer in the blank cuvettes. Emission was measured immediately after the addition of the last reactant and rapid mixing. Spectra were corrected for background fluorescence (sample minus blank). The excitation wavelength was set at 286 nm, and 5 mm-wide cuvettes were used to decrease the absorption. When necessary, corrections for absorption screening were made in accordance with Parker (1968), with factors never exceeding 1.2. The absorbance at the emission wavelength was always negligible. Fluorescence quenching of treated and untreated AP (0.18 mg/ml) was performed as described by Lehrer (1971). Iodide was maintained in the reduced state by the addition of 30 mM- $\text{Na}_2\text{S}_2\text{O}_3$, and KCl was used to compensate for the concentration of GndHCl and KI. The excitation was set at 286 nm for the above-mentioned reasons.

The light-scattering determinations at 90° angle were carried out with the LS5 spectrofluorimeter. After filtration with 0.45 μ m-pore-size Millipore membranes, the light-scattering intensity of the protein solution (0.2 mg/ml) was examined at 362 nm, following the procedure of Hand & Somero (1982). Each intensity value (in arbitrary units) was the result of automatic subtraction of blank scatter (GndHCl in buffer), which was significant, from that obtained soon after adding the protein to the same amount of inhibitor and rapid mixing.

Absorbance, fluorescence and light-scattering experiments were done at least in duplicate at $37 \pm 0.1^\circ\text{C}$.

Reversibility experiments

Protein solutions were treated with low (0.1 M) and high (1.5 M) concentrations of GndHCl. The former sample was rapidly submitted to dialysis in a collodion sac for 96 h at 25°C with five changes of 0.1 M-Tris buffer, pH 9.9, containing 0.15 mM- MgCl_2 ; the latter sample was preincubated with the modifier until AP activity was undetectable. Then both solutions, dialysed as just described, were separately gel-filtered on a column (2.5 cm $\times$ 70 cm) of Sephadex G-250 (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM-Tris buffer, pH 8.6, in accordance with Fosset *et al.* (1974) and eluted with a linear gradient of NaCl (0–0.05 M) in the same buffer. Flow rate was adjusted to approx. 0.30 ml/min, and 1.5 ml fractions were recovered with a SuperRac fraction collector from LKB (Bromma, Sweden). A control protein solution underwent the same procedures.

RESULTS AND DISCUSSION

Treatment with moderate amounts of GndHCl modifies intestinal AP activity with a biphasic effect, an instantaneous and a time-dependent one (Fig. 1). The ordinate intercept value, representing the 'time-zero' activity, ranges between 120% and 35% of the control. Instantaneous activation is found up to 1.0 M-GndHCl, after which an increasing inhibition can be observed. The

Fig. 1. Modulation of intestinal AP catalytic activity by GndHCl

Enzyme samples (0.004 mg/ml) were assayed at 37°C for various periods of time. Activity is expressed as percentage of the control (AP without GndHCl) at each concentration of the modifier (logarithmic scale) versus time. The ordinate intercept value is considered to be the 'time-zero' activity.

Fig. 2. Difference absorption spectra of intestinal AP treated with GndHCl

Absorbance (A) of AP alone was subtracted from that of AP treated with 0.05 M- (···), 0.5 M- (■—■), 0.9 M- (○—○), 1.1 M- (— · —) and 1.5 M- (—) GndHCl. Inset: shift of the wavelength of maximum absorbance in the GndHCl-treated protein. Other conditions were as described in the Experimental section.

time-dependent inactivation follows pseudo-first-order kinetics whose rate constants increase with inhibitor concentration. The difference absorption spectra, obtained after rapid mixing of the protein with the modifier, show a positive peak around 300 nm, preceded by a deep negative valley at about 260 nm (Fig. 2), which are suggestive of a change in tyrosine exposure, besides indicating contributions from variation of environment or ionization of tryptophan residues, phenylalanine residues and probably also disulphide groups (Donovan, 1969).

The interaction of the denaturant with the protein can be considered responsible for the concomitant red-shift of the absorption peak (Fig. 2 inset), due to change of solvent polarizability (Yanari & Bovey, 1960).

GndHCl was found to produce an immediate quenching and a red-shift of AP emission (Fig. 3). Fluorescence difference spectra present two peaks (not easily distinguished below 0.2 M- and above 1.0 M-guanidine): the first one at the protein maximum around 341 nm and a new one at about 328 nm, the latter peak suggesting an involvement of internal fluorophores. Separate effects on

Fig. 3. Difference fluorescence spectra of intestinal AP treated with GndHCl

Emission (F) of the protein treated with 0.10 M- (·····), 0.25 M- (----), 0.5 M- (—) and 1.1 M- (----) GndHCl was subtracted from that of the protein alone. Excitation was set at 286 nm, and slitwidths were set at 5 nm. Intensity is given in arbitrary units. Inset: Stern-Volmer plot of AP treated with GndHCl. Other details were as described in the Experimental section.

Fig. 4. Emission quenching by iodide of the GndHCl-modified AP

The Figure shows the fluorescence of AP alone minus that of protein treated separately with 0.25 M-KI (—) and with 1.0 M-GndHCl (----), and the fluorescence of GndHCl-treated AP minus that of protein with GndHCl and KI (○—○). Inset: Lehrer plot of KI quenching. Protein was treated with no (○), 0.25 M- (★) and 1.0 M-GndHCl (■). Other details were as described in the legend to Fig. 3 and in the Experimental section.

the emission of buried and exposed groups are illustrated by a non-linear Stern–Volmer plot (Fig. 3 inset) showing a break-point at about 0.5 M-GndHCl, below and above which the apparent quenching constants are 0.35 M^{-1} and 0.05 M^{-1} . The different efficiencies for the fluorophore classes can be ascribed to selective quenching of certain tryptophan residues, i.e. easily accessible residues are greatly depleted at low guanidine concentrations, whereas at higher ones those having lower quenching constants become dominant (Eftink & Ghiron, 1976). Additional experiments with iodide show that its effect is limited to groups emitting at longer wavelengths and less perturbed by guanidine (Fig. 4). The accessibility fraction, $(f_a)_{\text{eff.}}$, as calculated from Lehrer (1971) modified plots (Fig. 4 inset), is abruptly changed, reaching a minimum at about 0.5 M and rising again afterwards (Table 1), suggesting that the internal fluorophores are surfaced by the unfolding process. The reverse behaviour of quenching constant, $(K_Q)_{\text{eff.}}$, which increases at 0.25 M-GndHCl, could be explained by an enhanced proximity of iodide to fluorophores (Lehrer, 1971) in a collapsed structure. The return to the value typical of the native protein may imply that even when an ‘open’ partially unfolded conformation is reached the new structure still remains rather difficult to be penetrated by the ligand.

The decrease in scatter intensity below 0.25 M-GndHCl (Fig. 5a) is in agreement with the induction of a collapsed conformation that turns into a relaxed ‘open’ form of increasing size between 0.25 and 1.0 M, above which still another distinct state is presumed, probably due to a loss of intramolecular constraints (Hand & Somero, 1982).

The existence of several protein conformations is consistent with the behaviour of the differential absorbance, which increases especially after the addition of 0.5 M-GndHCl with small swings below 0.25 M and a slight decrease above 1.0 M (Fig. 5b). On the other hand, this assumption can also explain the emission data. In fact, the $\Delta F_{\text{red}}/\Delta F_{\text{blue}}$ fluorimetric ratio $[\Delta(F_{328} - F_{348})/\Delta(F_{328} - F_{308})]$, calculated from the values of the difference fluorescence spectra (see Fig. 3), shows a generally downward course with several turning points, at about 0.05 M-, 0.25 M- and 1.0 M-GndHCl, stressing the rapid change in protein optical properties.

The time changes of absorbance (at 278 nm), fluorescence (at 341 nm) and enzymic activity appear to differ (Fig. 6) when intestinal AP is treated with 1.1 M-GndHCl. The settling of the spectral properties after a big instantaneous jump could reflect the stabilization of the structural alterations that lead to the progressive catalytic

inhibition. At low concentrations (0.1 M-GndHCl) such modifications are difficult to follow because of the low power of the detection techniques employed. Moreover the use of activating concentrations of modifier (0.1 M) does not produce an irreversible conformational transition because the instantaneous jump (positive in the absorbance, negative in the fluorescence) is reversed by dialysis and gel filtration. However, the spectral and kinetic changes of the protein treated with 1.5 M-GndHCl are not reversible by dialysis. The modified AP indeed does not recover any activity and its spectral changes also persist after gel filtration, suggesting that a stronger derangement of molecular structure is produced.

Dissociation, aggregation or precipitation of the protein at higher concentrations of GndHCl (i.e. above 1.0 M), which have been already reported but only with much more elevated GndHCl concentrations (Schlesinger, 1965; Kadner *et al.*, 1968; Fosset *et al.*, 1974), should be excluded since the gel-permeation profiles of the treated and untreated protein are similar.

The effect of variation of pH, which is known to affect protein conformational stability (Ogasahara & Hamaguchi, 1967; Heitz & Brand, 1971), was also investigated. The employment of 0.9 M-GndHCl induces

Fig. 5. Comparison between the catalytic and optical properties of intestinal AP treated with GndHCl

‘Time-zero’ activity of the modified enzyme (O), expressed as percentage of the control, and the intensity of light-scattering (I_{LS} , ★), expressed in arbitrary units, are shown in (a). In (b) are displayed absorbance at 295 nm minus that at 255 nm of the treated enzyme $[\Delta(A_{295} - A_{255})]$, (O) and the fluorimetric ratio $(\Delta F_{\text{red}}/\Delta F_{\text{blue}})$, (★), calculated as explained in the Results and discussion section. Other conditions were as described in the legends to Figs. 1, 2 and 3 in the Experimental section.

Table 1. Fluorescence quenching by iodide of GndHCl-treated AP

Values were calculated in accordance with Lehrer (1971). Other conditions were as described in the legend to Fig. 2.

[GndHCl] (M)	$(f_a)_{\text{eff.}}$	$(K_Q)_{\text{eff.}}$ (M^{-1})
0	0.57	7
0.25	0.35	38
0.5	0.32	17
1.0	0.43	12

Fig. 6. Time-dependence of the catalytic activity (▲), absorbance (●) and fluorescence (★) of intestinal AP under treatment with 1.1 M-GndHCl

The values are expressed as percentages of the change observed in the treated protein (▲, ● and ★) and in the control (△, ○ and ☆) at the end of the time span considered.

Fig. 7. Effect of pH on the optical properties of intestinal AP treated with 0.9 M-GndHCl

(a) Difference absorption spectra (treated minus untreated AP) at pH 8.6 (---), pH 9.6 (·····), pH 10.0 (—) and pH 11.1 (---). (b) Difference fluorescence spectra (untreated minus treated AP) at pH 8.6 (---), pH 9.6 (·····), pH 10.0 (—) and pH 11.1 (---). Other conditions were as described in the legends to Figs. 2 and 3.

a blue-shift of the protein absorption, an effect that is abruptly reversed above pH 9.6 (Fig. 7a). A greater fluorescence deactivation is also found above this pH value, after which the emission difference spectra show the second quenching peak at 328 nm (Fig. 7b). Moreover, the 'time-zero' activity of treated AP is higher than the control between pH 8.4 and 9.9 and decreases progressively thereafter (Fig. 8a), paralleling the behaviour of the absorbance ratio at 294 nm (α), which is higher than unity in the pH range 8.6–9.6, with a minimum at pH 10.6 and a small increase above that pH (Fig. 8b). In more alkaline conditions the fluorescence intensity strongly decreases with respect to the control (Fig. 8c), with the emission maximum shifting to longer wavelengths.

Fig. 8. Dependence on pH of catalytic activity, absorbance and fluorescence of intestinal AP treated with 0.9 M-GndHCl

(a) Control activity (●) and 'time-zero' activity of the treated enzyme (○). (b) Ratio of the absorbance at 294 nm of the treated to that of the untreated protein (α , ●) and difference (in nm) of the peak wavelength of the treated minus that of the untreated protein ($\Delta\lambda_{\max}$, ○). (c) Differences in the intrinsic fluorescence at 341 nm (ΔF_{341} , ●) and in the emission peak wavelength ($\Delta\lambda_{\max}$, ○) of the treated that of the untreated protein (α , ●) and value. Experiments were performed as described in the legend to Fig. 1 in (a), in the legend to Fig. 2 in (b) and in the legend to Fig. 3 in (c).

These results indicate that at a lower pH a relatively constrained conformation is present, which can be disrupted by higher GndHCl concentrations in order to produce the observed activated structure. When an 'open' conformation is reached above pH 9.6 (Reynolds & Schlesinger, 1968), as also suggested by the shift of the emission and absorption maximum (Figs. 8*b* and 8*c*), the inhibited form is rapidly induced. The spectral modifications with pH, concurrent with the changes in activity, can then be regarded as documenting the impact of the denaturant with functionally important regions of the dimeric enzyme, for example the folded corner that is involved in the subunit interface and the proximal active centres (Wyckoff *et al.*, 1983; Sowadski *et al.*, 1985; Kam *et al.*, 1985). Guanidinium ions, by way of changing the respective environments and/or location of internal and superficial residues in a fluid structural matrix, may then involve particularly flexible and easily accessible groups at or near the active sites, as already found by Tsou (1986) for creatine kinase. The increased proximity and reactivity provoked by the interaction of few guanidine molecules determine activation, which at least in part could be sustained by guanidine phosphate-cleaving action (Lewin, 1974), a rate-limiting step (Lazdunski *et al.*, 1971). Very shortly thereafter, the intramolecular constraints necessary for structure maintenance and for the rearrangement of the partially denatured forms are involved in a time-dependent manner, so that at high guanidine concentrations the first phase is short-lived and the transition so fast that the activation cannot be observed. Inhibition appears after that conformational shuttling, which is considered the driving force of AP kinetic mechanism (Lazdunski *et al.*, 1971), is impaired and random formation predominates. The detection of at least three distinct phases in absorbance, fluorescence and light-scattering indicates that the system must contain different protein conformations (Dolgikh *et al.*, 1981). A three-state model (native, activated/inhibited and inactivated) is probably too simple to describe all the results, but it can provide a suitable basis for the interpretation of the kinetic events that occur upon treating intestinal AP with moderate concentrations of guanidine.

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