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The relationship between the optical properties and the kinetic behaviour of ascorbate-inhibited alkaline phosphatase

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Aromatic residues of bovine kidney alkaline phosphatase appear to be involved in the interaction with ascorbate, as shown by the strong quenching of intrinsic fluorescence and absorption. Difference u.v.-absorption spectra clearly indicate that conformational changes also occur. The pH value at which the greatest fluorescence deactivation is found is close to that necessary for optimal catalytic activity and for maximal inhibition by ascorbate. A protective effect against ascorbate is afforded by P_i. Time profiles of inactivation on one side and of absorbance and emission quenching on the other display opposite behaviours. Attempts to reverse the effects by the use of KOH fail to restore enzyme activity or to modify the spectral effects of ascorbate. The protein alterations are related, directly or indirectly, to the enzyme active centre and can be probably ascribed to the redox and chelating properties of ascorbate.

INTRODUCTION

Ascorbate is known to inhibit or stimulate the activity of various enzymes (Iwata *et al.*, 1979; Skotland & Ljones, 1980; Ramsay *et al.*, 1981). A strong inhibition of the activity of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) induced by ascorbate is accompanied by perturbations of protein spectral characteristics (Miggiano *et al.*, 1983; Martorana *et al.*, 1983, 1984). Studies on intrinsic fluorescence and absorption can yield valuable information on the reactions of enzymes with substrates, inhibitors and other effectors (Blomquist, 1967; Lasser & Feitelson, 1971; Heitz & Brand, 1971; Ramsay, 1982). The relationship between the optical properties and the catalytic aspects of the interaction of alkaline phosphatase with ascorbate with particular regard to effector concentration, time-dependence, pH-dependence, presence of P_i and effect of KOH is discussed in the present investigation.

EXPERIMENTAL

Materials

Alkaline phosphatase from bovine kidney (M_r 190000) and ascorbic acid sulphate potassium salt dihydrate were purchased from Calbiochem (La Jolla, CA, U.S.A.); L(+)-ascorbic acid and Tris were from Merck (Darmstadt, West Germany); *p*-nitrophenyl phosphate disodium salt was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); KOH and $Na_2HPO_4,2H_2O$ were from Carlo Erba (Milan, Italy). All compounds were dissolved in 1.0 M-Tris/HCl buffer, pH 8.0, which was preferred on kinetic considerations (Wilson & Cyr, 1964; Trentham & Gutfreund, 1968) and for maintaining constant pH and ionic strength of the medium. All other chemicals were of the best quality available.

Methods

The commercial enzyme was further purified by chromatography with DEAE-Trisacryl M (LKB, Bromma, Sweden) and Sephadex G-200 (Pharmacia, Uppsala, Sweden) in accordance with Cathala *et al.* (1975), with the first four steps omitted. Two peaks with phosphatase activity were isolated and recovered with a SuperRac fraction collector (also from LKB). Only the fractions of the first peak with the highest activity were pooled and used in the prosecution of the study. Purity of the enzyme preparation was checked by polyacrylamide-disc-gel electrophoresis in the presence of SDS in accordance with Weber & Osborn (1969) in a Quickfit and Quartz (Stone, Staffs., U.K.) apparatus. Only one band was observed. The enzyme preparation had a specific activity of 1740 units/mg, 1 unit being the amount of enzyme that catalyses the formation of 1 μmol of *p*-nitrophenol/min in 50 mM-Tris/HCl buffer, pH 9.8, containing 2.0 mM-$MgCl_2$, at 37 °C, monitored at 404 nm. Protein concentration was calculated from the absorption at 278 nm by using $A_1^{1\%}{}_{cm} = 7.9$ (Cathala *et al.*, 1975).

Fluorescence spectroscopy was performed in an LS5 Luminometer (Perkin–Elmer, Beaconsfield, Bucks., U.K.) as described previously (Martorana *et al.*, 1983, 1984). Excitation wavelengths were set at 277 nm, corresponding to the alkaline phosphatase absorption peak (Martorana *et al.*, 1983), and at 296 nm, where tryptophan fluorescence is selectively elicited and the absorbance of ascorbate (Karayannis *et al.*, 1977) is minimal. In our test conditions ascorbate showed ϵ_{277} and ϵ_{296} values of 8.194 and 1.157 $mM^{-1} \cdot cm^{-1}$ respectively. Absorbance was further decreased by using cuvettes with effective light-paths of 0.25 cm. Fluorescence intensity was finally corrected for inner-filter effects as described by Parker (1968), when A was $\geqslant 0.1$, recalculation of the intensity values being made with correction factors < 2.0. The absorbance at the emission wavelength was always negligible. Experimental conditions of fluorescence titrations were as follows: 250 μl of the protein solution in the sample cuvettes and 250 μl of the buffer in the blank cuvettes were added to 250 μl of buffer containing different ascorbate concentrations. Fluorescence difference spectra were obtained

by subtracting the emission of the untreated from that of the treated protein.

Absorbance was measured with an HP 8450A UV-Vis spectrophotometer (Hewlett–Packard, Palo Alto, CA, U.S.A.). Measurements were taken well inside the photometer response range, with final absorbance never exceeding 2.5. Absorption spectra were obtained by reading the test cuvettes, containing the protein and a fixed amount of the modifier(s), against a blank consisting of exactly the same amount of the modifier(s) only. Difference spectra were obtained by automatic subtraction of the spectrum of the untreated protein from that of the treated protein. Controls were performed with reference and measurement cuvettes both containing the same solutions (reagent or water or buffer or ascorbate or protein or the complete mixture), whose difference spectra were practically identical with the baseline. All spectra were also stored and retrieved for additional processing when necessary.

Catalytic assays and dialysis were performed as described by Miggiano *et al.* (1984).

Absorbance, fluorescence and rate measurements were done at 37(±0.1) °C, with all the experiments carried out at least in duplicate.

Plotting was performed with the aid of an Olivetti (Ivrea, Italy) M40 computer.

RESULTS

Ascorbate decreases intrinsic fluorescence and absorbance of alkaline phosphatase. No modification is observed in the shape of the emission difference spectra (Fig. 1). Differential excitation at 277 and 296 nm clearly reveals the tyrosine contribution and demonstrates that the ascorbate quenching action is exerted virtually throughout the spectrum. The difference spectrum between the absorbance of the enzyme exposed to ascorbate and that of the free enzyme shows a large negative peak near 271 nm, red-shifted at higher effector concentration, and a small positive peak around 288 nm, which appears and increases at elevated ligand/protein molar ratios (above 200:1) (Fig. 2). Fluorescence and absorbance changes evoked on alkaline phosphatase by different amounts of ascorbate are reported in the insets of Figs. 1 and 2. Half-maximal difference in absorbance is obtained with 0.18 mM-ascorbate, and 0.28 mM-ascorbate yields half-maximal quenching with 277 nm excitation. Ascorbic acid sulphate ($\epsilon_{277} = 1.295\ mM^{-1} \cdot cm^{-1}$) has no effect on the protein fluorescence and the absorbance even at very high concentrations (up to 1.23 mM).

The greatest fluorescence deactivation induced by ascorbate was found at about pH 10.0 (Fig. 3).

P_i, which shields enzyme from ascorbate inhibition (Miggiano *et al.*, 1984), was also tested. The non-covalent enzyme–P_i complex, formed in alkaline media (Hull *et al.*, 1976), displays an emission band overlapping that of the unliganded enzyme. High concentrations (150 mM) of P_i are particularly effective in the 310–360 nm emission range (Fig. 4). The different protective action of P_i observed with 277 and 296 nm excitation indicates that it affords a better shielding of deep-emitting fluorophores.

P_i alone does not exert any significant effect on alkaline phosphatase absorbance (Fig. 5, spectrum a). However, the difference spectrum between the enzyme–P_i

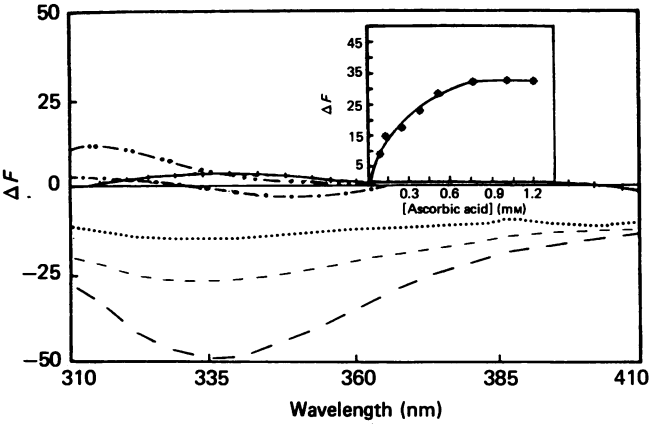

Fig. 1. Ascorbate quenching of the intrinsic fluorescence of alkaline phosphatase

Fluorescence difference spectra of 0.88 μM-alkaline phosphatase treated with 0.28 mM- (· · · · · ·), 0.85 mM- (– – – –) and 1.70 mM- (——) ascorbate minus the emission of untreated enzyme (excitation at 296 nm), or treated with 1.23 mM-ascorbic acid sulphate (–+–+–) minus the emission of untreated enzyme (excitation at 277 nm). The effects of differential excitation (277 nm minus 296 nm) on the emission of untreated alkaline phosphatase (· · –· · –) and of alkaline phosphatase treated with 0.28 mM-ascorbate (–· –· –) are also shown (intensities equalized at 370 nm). Inset: changes of fluorescence differences (ΔF) of alkaline phosphatase produced by the interaction with ascorbate at various concentrations (emission observed at 335 nm with excitation at 277 nm). Instrumental conditions were: excitation and emission slit 5 nm; scale factor 1; response factor 2; automatic zero; scan speed 240 nm/min. Fluorescence intensities are expressed in arbitrary units. All other details are given in the Experimental section.

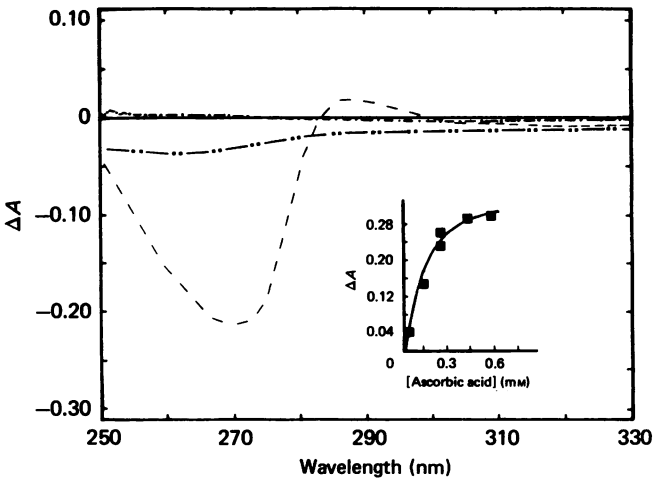

Fig. 2. Effect of ascorbate on the absorbance of alkaline phosphatase

Difference absorption spectra of 0.88 μM-alkaline phosphatase treated with 0.07 mM-(· · –· · –) and 0.28 mM-(– – – –) ascorbate and 1.23 mM-ascorbic acid sulphate (–· –· –· –) minus the free enzyme. Inset: changes of absorption differences (ΔA) at the peak wavelength produced by the interaction of alkaline phosphatase with various concentrations of ascorbate. Conditions are as reported in the Experimental section.

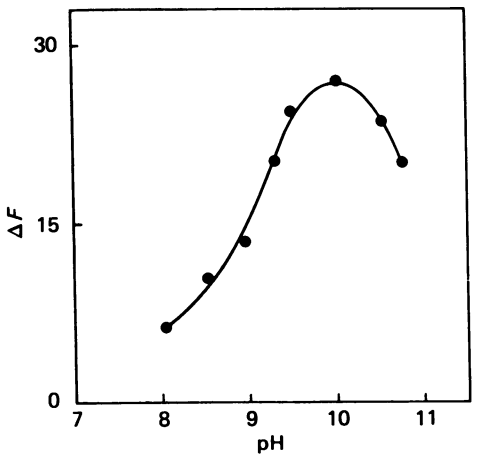

Fig. 3. Influence of pH on the quenching by ascorbate of fluorescence emission by alkaline phosphatase

ΔF represents the difference in the fluorescence between 0.88 μM-alkaline phosphatase treated with 0.28 mM-ascorbate and untreated enzyme both at the same pH value. The experiments were performed in 0.5 M-Tris/HCl buffer prepared and maintained at each different pH. Excitation was set at 296 nm, where ascorbate absorbance ranged from 1.157 to 1.716 $mM^{-1} \cdot cm^{-1}$. The other experimental and instrumental conditions are as indicated in Fig. 1 legend.

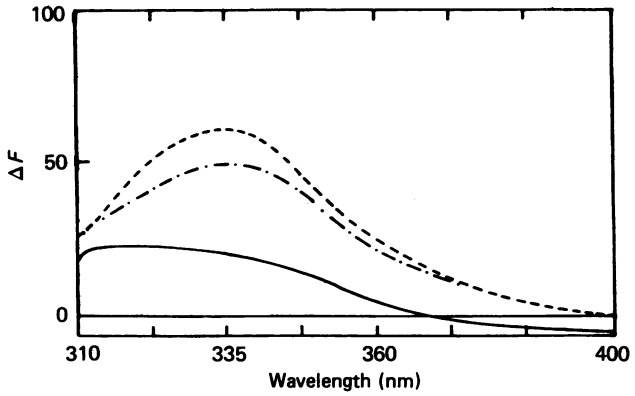

Fig. 4. Effect of P_i on the fluorescence of ascorbate-treated alkaline phosphatase

Difference spectra between the fluorescence of 0.88 μM-alkaline phosphatase treated with 150.0 mM-P_i and then with 0.28 mM-ascorbate, and that of the enzyme treated with 0.28 mM-ascorbate only; excitation was first set at 296 nm (----) and then at 277 nm (-·-·-·). ——, Difference in emission of the protein treated with both compounds and successively excited at the two wavelengths (277 nm minus 296 nm; values normalized at 370 nm and multiplied 3-fold). Other instrumental conditions are as indicated in Fig. 1 legend.

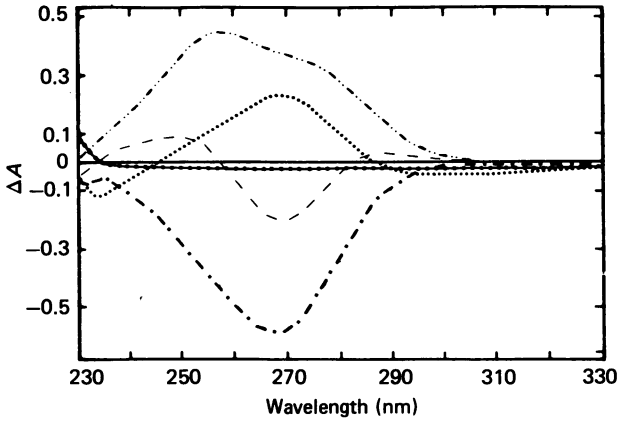

Fig. 5. Effect of P_i on the absorbance of ascorbate-treated alkaline phosphatase

Difference absorption spectra: spectrum a, between 4.7 μM-alkaline phosphatase treated with 30.0 mM-P_i and untreated enzyme (——); spectrum b, between alkaline phosphatase treated with 0.28 mM-ascorbate and untreated enzyme (-·-·-); spectrum c, between alkaline phosphatase, treated first with 30.0 mM-P_i and then with 0.28 mM-ascorbate, and enzyme treated with 30.0 mM-P_i only (----); spectrum d, spectrum c minus spectrum b (··-··-); spectrum e, as for spectrum d except that alkaline phosphatase was treated first with 0.28 mM-ascorbate and then with 30.0 mM-P_i (·····).

complex treated with ascorbate and the same untreated shows a negative peak near 270 nm and a positive one near 284 nm (Fig. 5, spectrum c). This pattern is quite different from the one observed after the addition of ascorbate alone (Fig. 5, spectrum b). In fact the difference spectrum between the P_i-bound enzyme and the free enzyme, each then treated with ascorbate, shows a positive absorption difference at all wavelengths, with a maximum at 258 nm with $\Delta A = 0.462$ (Fig. 5, spectrum d). If added after ascorbate, P_i loses some effectiveness, the peak is shifted to 268 nm and $\Delta A_{max.}$ is lowered to about 0.250 (Fig. 5, spectrum e). Intrinsic fluorescence, absorbance and catalytic activity of ascorbyl-enzyme were monitored for 1 h concurrently with the stability of ascorbate preparations (Fig. 6). As already reported (Meucci *et al.*, 1985), the presence of enzyme does not modify the ascorbate autoxidation rate to any great extent (inset of Fig. 6). The quenching produced by ascorbate (excitation at 300 nm, where ascorbate does not present any significant absorption ($A < 0.1$), is instantaneous, but alkaline phosphatase fluorescence is partially restored with time. The action of dehydroascorbate formed during ascorbate autoxidation has much less effect on the fluorescence quenching (Martorana *et al.*, 1984) and can be disregarded. The difference in absorbance $[\Delta(A_{288} - A_{271})]$ in the difference spectrum between treated and untreated alkaline phosphatase changes in a similar manner. Inactivation instead follows a pseudo-first-order course and is not even partially reversed by dialysis, as found previously (Miggiano *et al.*, 1984).

The activity and the fluorescence intensity of the enzyme treated with ascorbate and KOH were monitored for 2 h (Table 1). The oxidant, used in order to antagonize the effects of ascorbate (Veazey & Nieman, 1980), also displays a quenching and inhibitory effect. Upon the addition of both compounds a diminished inactivation is observed, though ascorbate quenching is only slightly affected. After the enzyme has been treated with ascorbate for 2 h and then dialysed, KOH produces a stronger quenching and inhibition, effects no return of intrinsic fluorescence and fails to restore any activity at all.

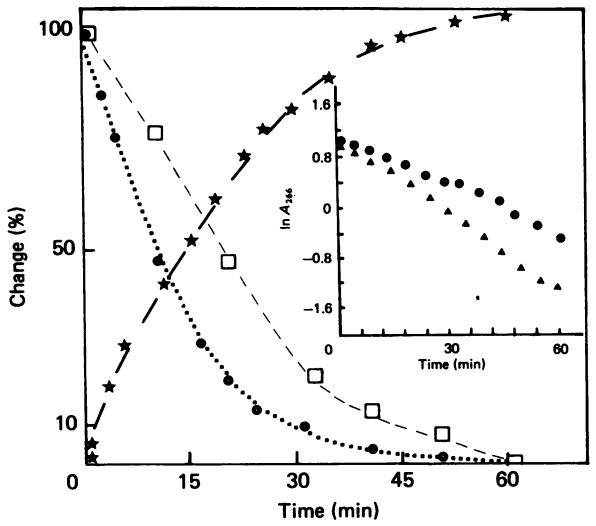

Fig. 6. Time-dependence of the ascorbate effects on alkaline phosphatase activity, fluorescence and absorbance

Activity (★) of 0.84 μM-alkaline phosphatase was monitored at various time intervals during treatment with 0.56 mM-ascorbate. Fluorescence intensity (○) of 0.79 μM-alkaline phosphatase at 335 nm in the presence of 0.28 mM-ascorbate (excitation 300 nm) was noted every 6 s. $\Delta(A_{288}-A_{271})$ (□) of 0.79 μM-alkaline phosphatase with 0.28 mM-ascorbate was measured every 2 min. For the sake of clarity not all the experimental points are presented. Measurements were started soon after the addition of the last reactant. The data are expressed as percentages of the change observed in the time span considered. Inset: decay patterns of 0.28 mM-ascorbate alone (▲) and with 0.79 μM-alkaline phosphatase (●), obtained by monitoring the decrease of absorbance at 266 nm, the absorption peak of ascorbate and of the ascorbate–enzyme complex in our conditions. For other details see the legends of Figs. 1 and 2 and the Experimental section.

DISCUSSION

Interaction between intrinsic chromophores and ligands bound to specific or less-specific protein regions can be a particularly sensitive measure of conformational changes, which may be reflected in optical modifications due to environment perturbation (Yielding & White, 1976). The spectroscopic changes observed in the interaction between ascorbic acid and alkaline phosphatase can be ascribed to the effect of the anionic reduced form, readily produced in alkaline conditions and actually considered to be the active compound (Swartz & Dodd, 1981), directly on aromatic residues or through vicinal binding to other side groups containing labile hydrogen atoms (Lewin, 1976). A proton-transfer mechanism can explain the quenching of fluorescence (Saito *et al.*, 1984). The shape of the spectra, predominantly due to tryptophan emission (Teale, 1960), does not appear to be significantly changed. On the other hand, the red-shift of the absorption maximum suggests a modification in which aromatic side chains become buried in the supposedly more hydrophobic interior (Donovan, 1969). The absorption changes in the 271 nm and 285 nm regions are assigned mainly to tyrosine and partly to tryptophan (Donovan, 1969; Reynolds & Schlesinger, 1969; Grosse *et al.*, 1977). The involvement of tyrosine in the catalytic process of human placental alkaline phosphatase has been suggested by Chang & Chang (1984) on the basis of chemical-modification studies. Although the exact location of tyrosine and tryptophan residues and their importance for the catalytic function of bovine kidney alkaline phosphatase are not known, an interrelationship between spectroscopic and kinetic phenomena is, in our opinion, difficult to exclude. High concentrations of ascorbic acid sulphate, in fact, have no effect at all on enzyme activity (Miggiano *et al.*, 1984) and spectral properties, whereas dehydroascorbate weakly quenches and inhibits (Martorana *et al.*, 1984).

Moreover, the similar patterns of pH-dependence for activity, inactivation (Miggiano *et al.*, 1984) and optical modifications suggest that the same conformation is the most suitable for catalysis, inhibitor binding and quenching of fluorescence.

Active-site-directed substances, such as substrate and P_i itself, can completely counteract the inhibitory effects of ascorbate (Miggiano *et al.*, 1984). Particularly when P_i is added first, the red-shifted and positive differential absorption and the greater extent of shorter-wavelength emission, observed with differential excitation, are consistent with a more efficient shielding of fluorophores in the more hydrophobic protein interior (Lakowicz,

Table 1. Effect of KOH on alkaline phosphatase–ascorbate interaction

Experimental conditions were as indicated in Fig. 6 legend. Values represent the means ± S.E.M. for five determinations. Abbreviation: AP, alkaline phosphatase.

	Activity (units/l)		Fluorescence (arbitrary units)	
	Zero time	After 120 min	Zero time	After 120 min
AP, untreated	293±4	290±5	78±3	78±3
AP with 0.56 mM-ascorbate	291±4	223±4	58±2	70±2
AP with 20 mM-KOH	287±4	225±4	62±2	60±2
AP with 0.56 mM-ascorbate and 20 mM-KOH	268±4	269±4	58±2	65±2
AP with 20 mM-KOH, after 120 min with 0.56 mM-ascorbate	211±4	105±3	50±2	50±2

1983). Some hindrance and interference between ascorbate and P_i can then be inferred at the level of internal groups in, or adjacent to, the active-site cleft (Coleman & Gettins, 1983), similarly to what has already been suggested for other anions, such as MnO_4^- (Thomas & Kirsch, 1980).

The diminished inactivation and the lack of cumulative effect on fluorescence when ascorbate and KOH are added together can be interpreted in terms of both compounds neutralizing each other before affecting alkaline phosphatase. The failure of the oxidant to reverse the action of ascorbate, the effect of KOH alone and the different time behaviours displayed by optical properties and inhibition suggest that the intimate mechanism of ascorbate-induced changes cannot be entirely ascribed to a redox-state modification. It may also depend on subtle but permanent alterations of zinc reactivity, probably due to the chelating action of ascorbate (Lewin, 1976), as already documented for alkaline phosphatase with other metal-complexing agents by absorption and c.d. spectra (Reynolds & Schlesinger, 1969) and n.m.r. studies (Hull & Sykes, 1976).

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