The Influence of Chloride and other Univalent Inorganic Anions on the Visible-Absorption Spectrum of Aspartate Aminotransferase

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1. The influence of Cl^- , Br^- , NO_3^- and F^- ions on the visible-absorption spectrum of deionized aspartate aminotransferase was investigated. 2. Except for F^- , these anions caused an increase of the extinction at $430\text{ m}\mu$ with a concomitant decrease of that at $362\text{ m}\mu$. 3. The affinity constants for Cl^- and NO_3^- ions were calculated by a procedure based on the assumption that the anion stabilizes the protonated form of the enzyme chromophore (λ_{max} , $430\text{ m}\mu$). 4. The true pK of the chromophore of the enzyme was found to be 5.25.

One of the characteristic features of aspartate aminotransferase (L - aspartate - 2 - oxoglutarate aminotransferase, EC 2.6.1.1) is its behaviour as a pH indicator. The enzyme solution, which is bright yellow (λ_{max} , $430\text{ m}\mu$) at pH 5.4, becomes colourless (λ_{max} , $362\text{ m}\mu$) with increasing pH (Jenkins, Yphantis & Sizer, 1959). The yellow colour of the enzyme was attributed to the addition of a single proton to the chromophore of the enzyme, and a pK value of 6.3 was calculated spectrophotometrically by Jenkins *et al.* (1959). At present the chromophoric group of aspartate aminotransferase is generally recognized in the aldimine linkage, $-\text{CH}=\text{N}-$, between the 4-formyl group of the coenzyme (pyridoxal 5'-phosphate) and the ϵ -amino group of a lysine residue on the apoenzyme (Fasella, 1967). Marino, Greco & Scardi (1965) observed that complete desalting causes a marked change in the visible-absorption spectrum of ox heart aspartate aminotransferase while the pH of the medium remains unchanged. After prolonged dialysis against water the enzyme solution shows two symmetrical absorption bands at 425 and $365\text{ m}\mu$ corresponding to the spectrum at pH 6.3, whereas after deionization the peak at $425\text{ m}\mu$ disappears with concomitant increase of the one at $365\text{ m}\mu$. This effect, which resembles what happens after an increase in pH from 6.2 to 7.7, can be partially reversed by addition of a neutral salt, such as sodium chloride. It was suggested that deionization of aspartate aminotransferase causes the removal of a counter-anion with subsequent decrease of the pK of the chromophore (Fasella, 1967).

The work reported in the present paper is an investigation of the influence of Cl^- and other univalent inorganic anions on the visible-absorption

spectrum of deionized aspartate aminotransferase. The ' α -form' of the pig heart enzyme (Martinez-Carrion *et al.* 1967) was used instead of the ox heart enzyme on which the effect of deionization was first observed. The ' α -form' from pig heart is more homogeneous than the ox heart enzyme, which seems to contain at least seven sub-forms not yet completely isolated and characterized (G. Marino & V. Scardi, unpublished work).

EXPERIMENTAL

Materials. The ' α -form' of pig heart aspartate aminotransferase was prepared essentially by the method of Martinez-Carrion *et al.* (1967) and deionized as described by Marino, Greco, Scardi & Zito (1966).

The salts NaCl , NaF , NaBr and NaNO_3 were all analytical-grade products from commercial sources.

Methods. Graded amounts of a concentrated salt solution containing the anion under assay were added to the deionized enzyme (0.1 mm) in a silica cuvette with an optical path of 0.5 cm ., and thoroughly mixed. After each addition the spectrum was recorded from 320 to $500\text{ m}\mu$ in a Beckman DK-2 spectrophotometer. To compensate for the dilution of the enzyme after each salt addition, the spectral changes were given as $\Delta E_{430}/\text{mg. of enzyme}$ (or $\Delta E_{362}/\text{mg. of enzyme}$). The enzyme concentration was evaluated spectrophotometrically at $278\text{ m}\mu$ by using $E_{1\text{cm}}^{1\%}$, 14.80, obtained from dry-weight measurements.

Salt concentrations reported in this paper are expressed as activities, γm ; the activity coefficients γ were taken from Robinson & Stokes (1955), and for molality values, m , lower than 0.1 they were calculated according to the Debye-Hückel equation:

$$\log \gamma = -Az_1z_2\sqrt{I}$$

where A is 0.509 at 25° .

The pH measurements were made in a Radiometer PH4 pH-meter.

Fig. 1. Effect of Cl^- ions on the absorption spectrum of the deionized aspartate aminotransferase. The spectra were traced from the original automatically recorded spectra. Final concentrations of Cl^- ions (added as NaCl) were 0 (arrow), 0.0088, 0.017, 0.039, 0.065, 0.107, 0.140, 0.215, 0.285 and 0.415 (expressed as activities, γm).

Scheme 1. For simplicity the functional groups CH_3 -, HO - and $-\text{CH}_2\cdot\text{O}\cdot\text{PO}_3\text{H}_2$ in positions 2, 3 and 5 respectively have been omitted.

RESULTS AND DISCUSSION

Addition of Cl^- , Br^- and NO_3^- ions to the deionized aspartate aminotransferase caused characteristic changes in the visible-absorption spectrum. No spectral change was observed with F^- ions, even when the sodium salt was added at concentration approaching saturation. Fig. 1 shows the effect of adding graded amounts of Cl^- ions to the deionized enzyme.

By assuming that the colour change, namely the increase of E_{430} with concurrent decrease of E_{362} , is merely due to the addition of one proton to the chromophoric group of the enzyme, the effect of the anions under assay might consist in stabilizing the protonated aldimine linkage, $-\text{CH}=\text{NH}^+$, as depicted in Scheme 1. It follows that the affinity

Fig. 2. Determination of the affinity constant K_A for Cl^- ions (see eqn. 1). The ordinate shows the reciprocal of ΔE_{430} resulting from the addition of graded amounts of Cl^- ions (as NaCl). The abscissa shows the reciprocal of the Cl^- ion concentration (expressed as activity, γm).

Fig. 3. Determination of the affinity constant K_A for Cl^- ions by plotting the reciprocal of the decrease in E_{362} resulting from the addition of graded amounts of Cl^- ions (as NaCl) against the reciprocal of Cl^- ion concentration (expressed as activity, γm).

of the anion, A^- , for the chromophoric group of the enzyme can be measured by:

$$K_A = \frac{[\text{En}_I][\text{A}^-]}{[\text{En}_{II}]} \quad (1)$$

where En_I is the non-protonated chromophore (colourless enzyme, $\lambda_{\text{max.}} 362 \text{ m}\mu$) and En_{II} the protonated enzyme (yellow enzyme, $\lambda_{\text{max.}} 430 \text{ m}\mu$). This expression ignores the proton, although it plays a fundamental role in the colour change of the enzyme solution. Owing to the absence of pH change, it is conceivable that the proton comes from the enzyme protein (see Scheme 1) rather than from the medium; a proton transfer from the medium to the aldimine linkage would cause a detectable pH change of the enzyme solution. The value of K_A was obtained by measuring the change in E_{430} and E_{362} , i.e. ΔE_{430} and ΔE_{362} , on addition of graded amounts of anion and plotting respectively $1/\Delta E_{430}$ and $1/\Delta E_{362}$ against $1/[\text{anion}]$; K_A is then

Fig. 4. Dependence of apparent pK of the chromophore of aspartate aminotransferase on the Cl^- ion concentration (expressed as activity, γm).

the reciprocal of the intercept of the plot on the abscissa (cf.Sizer & Jenkins, 1963). Figs. 2 and 3 illustrate this procedure. K_A values of 0.22 and 0.31 were calculated for Cl^- and NO_3^- ions respectively. With Br^- ions it was not possible to calculate the value of K_A by this procedure because of the non-linearity of the plot at low values of $1/[\text{Br}^-]$.

The change in extinction on complete protonation of the chromophore (i.e. the maximum ΔE_{430}) is given by the reciprocal of the intercept on the ordinate axis. This value is coincident with that obtained after dialysis of the deionized enzyme against 5M-sodium chloride or 5M-sodium nitrate.

The linearity of the plots shown in Figs. 2 and 3 indicates that the assumed stoichiometry of the equilibrium in Scheme 1 is formally correct.

It is noteworthy that the spectral change caused by anions can be reversed only by deionization, as dialysis against water is ineffective, probably as a consequence of a Donnan equilibrium.

The experiments reported in this paper clearly

demonstrate that the pK of the chromophore of the enzyme is influenced by the anion concentration. Hence a relation between the apparent pK value and the anion concentration should exist. On this assumption apparent pK values were calculated for each concentration of the anion by the following equation:

$$pK_{\text{app.}} = \text{pH} + \log \left(\frac{\alpha}{1 - \alpha} \right) \quad (2)$$

where pH is constant and equal to 6.5 and α is given by the ratio $\Delta E_{430}/(\Delta E_{430})_{\text{max.}}$ at each anion concentration. Fig. 4 shows the relation between apparent pK of the chromophoric group of aspartate aminotransferase and Cl^- ion concentration. Owing to the uncertainty of the extrapolation to zero concentration, a better extrapolated value of pK_0 was obtained by plotting $1/K_{\text{app.}}$ against [anion]. For Cl^- and NO_3^- ions a pK_0 value of 5.25 was found. This value represents the true pK of the chromophore of aspartate aminotransferase.

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