Effect of pH, Ionic Strength and Univalent Inorganic Ions on the Reconstitution of Aspartate Aminotransferase

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1. The effect of pH change on the reconstitution of aspartate aminotransferase (EC 2.6.1.1), i.e. the reactivation of the apoenzyme with coenzyme (pyridoxal phosphate and pyridoxamine phosphate), was studied in the pH range 4.2–8.9 by using three buffer systems at concentrations ranging from 0.025 to 0.1 M. 2. Although the profile of the reconstitution rate–pH curve in the range pH 5.2–6.8 (covered by sodium cacodylate–HCl buffer) reflects the influence of the H^+ concentration on the reconstitution process, the profile of the curve in the pH ranges 4.2–5.6 and 7.2–8.25 (covered respectively by sodium acetate–acetic acid and Tris–HCl buffers) appears to be influenced by the ionic strength of the buffer. 3. The reconstitution is also influenced by univalent inorganic ions such as halide ions and, to a lesser extent, alkali metal ions, which are known to alter the water structure.

The effect of pH change on the reconstitution of aspartate aminotransferase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1), i.e. the reactivation of apoenzyme with coenzyme, has not been extensively investigated hitherto. Meister *et al.* (1954) first reported that the reactivation of the pig heart apoenzyme, either with pyridoxal phosphate or pyridoxamine phosphate, was faster at pH 9.0 than at pH 6.0. This was not confirmed by Torchinsky (1963), who reported that in 0.125 M-phosphate the velocity of reconstitution gradually increases from pH 9.0 to 7.0. Scotto & Scardi (1964) measured the initial reconstitution velocity over a broad pH interval (pH 4.5–10.5) and observed that the pH–velocity curve showed a maximum at pH 6.0 and an S-shaped portion from pH 8.5 to 10.5 when pyridoxal phosphate was used as coenzyme; with pyridoxamine phosphate the pH–velocity curve showed no definite maximum in the acidic region and an S-shaped portion from pH 8.5 to 10.5. The discrepancy between these results and those of Torchinsky (1963) was probably due to the fact that the latter author actually measured the percentage of apoenzyme reactivated after a rather long incubation time (10–15 min) under conditions (37°C and excess of coenzyme) that did not allow observation of any variation of the reconstitution velocity after pH change. The results of Scotto & Scardi (1964) were also doubtful, owing to the use of a phosphate buffer system (McIlvaine, 1921), which inhibits the reconstitution (Banks *et al.*, 1963; Hayashi *et al.*, 1967). For a better evaluation of the reconstitution rate the effect of pH on the reactivation of both the pig heart apoenzyme (A. M. Greco, G. Marino & V. Scardi, unpublished work) and the ox heart apoenzyme (Scardi, 1968) was reinvestigated by using

(a) buffer systems containing no phosphate ions, (b) stoichiometric amounts of apoenzyme and coenzyme, and (c) low incubation temperature (0°C with pyridoxal phosphate, 14.5°C with pyridoxamine phosphate). The half-reconstitution time was measured instead of the initial velocity, since this can be done more accurately especially for very fast processes. The plot of the reciprocal half-reconstitution time against pH showed two optima (about pH 5.5 and 9) when pyridoxal phosphate was used as coenzyme, and only one optimum (about pH 9.5) with pyridoxamine phosphate (A. M. Greco, G. Marino & V. Scardi, unpublished work; Scardi, 1968). From these results an attempt was made to identify the functional groups involved in the binding of coenzyme to apoenzyme (Scardi, 1968). However, difficulties arose because of the possible influence of ionic strength and/or buffer ions on the position of the reconstitution rate maxima. Preliminary experiments with buffers containing dicarboxylate ions (Marino & Scardi, 1968) or with NaCl added to obtain constant ionic strength encouraged a systematic investigation on this aspect of the reconstitution.

In the present work the effects of pH, ionic strength and univalent inorganic ions on the reconstitution rate of pig heart aspartate aminotransferase are reported.

Experimental

Materials

The apoenzyme was obtained as previously described (Marino *et al.*, 1966) from the α -form of the

pig heart enzyme prepared by the method of Martinez-Carrion *et al.* (1967). The resolution calculated according to Scardi *et al.* (1963) was higher than 90%. The presence of 0.1 mM-GSH allowed the apoenzyme stock solution to be stored at +5°C for several days without affecting the reconstitution reaction. The apoenzyme concentration was measured spectrophotometrically at 278 nm by using an extinction coefficient $E_{1\text{cm}}^{1\%}$ of 14.40 (obtained on a dry-weight basis) and a minimum molecular weight of 47000.

Pyridoxal phosphate and pyridoxamine phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. All common chemicals were pure products from different commercial sources.

Methods

The reconstitution velocity was determined by measuring the activity of the holoenzyme formed on incubation of apoenzyme and coenzyme at room temperature ($18 \pm 1^\circ\text{C}$) for a fixed time: this was 7 s with pyridoxal phosphate as coenzyme, 30 s with pyridoxamine phosphate. Unless otherwise stated both forms of coenzyme were always present in a 20-fold excess with respect to the stoichiometric amount, so that reconstitution followed pseudo-first-order kinetics. Because of the very short incubation time, measurements were performed manually as follows. Buffer solution (25 μl) containing the apoenzyme (0.1–0.2 nmol) was added to 2 μl of a solution containing 2.0–4.0 nmol of pyridoxal phosphate (or pyridoxamine phosphate) on the flattened and bent end of a small plastic rod, which after exactly 7 s or 30 s respectively was dipped into a silica cuvette containing 2.7 ml of the reagent mixture for assaying the aspartate aminotransferase activity as described by Jenkins *et al.* (1959). Owing to the strong dilution of the incubation mixture, the reconstitution is substantially arrested, and the activity measured as oxaloacetate formed per unit time (i.e. as $\Delta E_{280}/\text{min}$) represents the amount of holoenzyme formed in 7 s or 30 s respectively, i.e. the velocity of reconstitution. For convenience reconstitution-velocity measurements have been multiplied by 10^3 . All measurements were carried out in a Beckman DB recording spectrophotometer equipped with a water-bath circulating system regulated at $26 \pm 0.1^\circ\text{C}$.

Results and Discussion

Effect of pH

The reconstitution of aspartate aminotransferase was studied over the pH range 4.2–8.8 by using the following 0.05 M buffer systems prepared as described by Dawson & Elliott (1959): sodium acetate–acetic acid, sodium cacodylate–HCl and Tris–HCl. These

contain no ions known to interfere with the reactivation of apoenzyme with coenzyme. Imine formation between Tris and pyridoxal phosphate (Metzler, 1957) may be neglected under the present experimental conditions.

With pyridoxal phosphate the reactivation of apoenzyme as a function of pH showed two maxima, at pH 5.5 and 8.25, in fairly good agreement with those previously reported (Scardi, 1968). In contrast, the reconstitution with pyridoxamine phosphate showed only one maximum, at pH 5.5, whereas from pH 7.4 to 8.8 its pH-dependence was represented by an S-shaped curve.

Effect of ionic strength

The three buffer systems used to cover the pH range 4.2–8.8 had the same molarity (0.05 M) but not the same ionic strength; except for the cacodylate buffer ($I = 0.05$), the ionic strength had values varying with pH. To conserve space, plots of I against pH for acetate and Tris buffers are not reported here. However, especially for high buffer concentration the variation of I with pH is marked, e.g. the I value of 0.1 M-acetate buffer changes from 0.02 to 0.09 by passing from pH 4.2 to 5.6. To obviate this, preliminary experiments were carried out in acetate and Tris buffers whose ionic strength had been adjusted to the same value with NaCl. Under these conditions the reconstitution rate, either with pyridoxal phosphate or pyridoxamine phosphate, showed no definite pH optimum, thus indicating a sensitivity to the ionic strength of the medium without otherwise excluding a possible effect of Na^+ and/or Cl^- ions, an effect that will be dealt with below. The reconstitution rate as a function of pH was then measured at four different concentrations of each buffer, namely 0.025, 0.05, 0.075 and 0.1 M. In cacodylate buffer the reconstitution rate appeared to be independent of the buffer concentration (i.e. ionic strength), but in acetate and Tris buffers a family of four curves was obtained (Figs. 1, 2 and 3). To separate the effect caused by pH change and that caused by ionic strength, the reconstitution rate at each pH value was plotted against the buffer ionic strength. With Tris buffer the plot was linear and extrapolation to zero ionic strength permitted calculation of the reconstitution rate at various pH values (Fig. 4); an optimum pH was found at 8.25 (see inset, Fig. 4), thus confirming the value reported above. With acetate buffer the plot was non-linear, so that no extrapolation was possible (Fig. 5). The reconstitution rate was then studied in a series of acetate buffers of the same ionic strength, with pH values ranging from 4.4 to 5.6. These were prepared from equimolar solutions of sodium acetate by adjusting the pH to the required value with acetic acid. The results of these experiments indicated that the reconstitution rate increases with

Fig. 1. Effect of pH on the velocity of reconstitution of aspartate aminotransferase with pyridoxal phosphate in acetate buffer

For details see the text. The following buffer concentrations were used: 0.1 M (○), 0.075 M (□), 0.05 M (△) and 0.025 M (●). The reconstitution velocity is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme and coenzyme for 7 s at room temperature ($18 \pm 1^\circ\text{C}$).

Fig. 2. Effect of pH on the velocity of reconstitution of aspartate aminotransferase with pyridoxal phosphate in cacodylate buffer

For details see the text. The following buffer concentrations were used: 0.1 M (○), 0.05 M (□) and 0.025 M (△). The reconstitution velocity is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme and coenzyme for 7 s at room temperature ($18 \pm 1^\circ\text{C}$).

Fig. 3. Effect of pH on the velocity of reconstitution of aspartate aminotransferase with pyridoxal phosphate in Tris buffer

For details see the text. The following buffer concentrations were used: 0.1 M (○), 0.075 M (□), 0.05 M (△) and 0.025 M (●). The reconstitution velocity is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme and coenzyme for 7 s at room temperature ($18 \pm 1^\circ\text{C}$).

Fig. 4. Effect of ionic strength on the velocity of reconstitution of aspartate aminotransferase with pyridoxal phosphate in Tris buffer

For details see the text. Buffer solutions at the following pH values were used: pH 7.50 (●), 7.75 (▲), 8.25 (□), 8.60 (△) and 8.9 (■). The inset shows a plot of v , the reconstitution velocity extrapolated to $I = 0$, against pH. The reconstitution velocity is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme and coenzyme for 7 s at room temperature ($18 \pm 1^\circ\text{C}$).

Fig. 5. Effect of ionic strength on the velocity of reconstitution of aspartate aminotransferase with pyridoxal phosphate in acetate buffer

For details see the text. Buffer solutions with the following pH values were used: pH 4.25 (Δ), 4.40 ($\circ$), 4.60 ($\bullet$), 4.75 ($\blacksquare$), 5.15 ($\square$) and 5.50 ($\blacktriangle$). The reconstitution velocity is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme and coenzyme for 7 s at room temperature ($18 \pm 1^\circ\text{C}$).

Fig. 6. Effect of ionic strength on the velocity of reconstitution of aspartate aminotransferase with pyridoxamine phosphate in Tris buffer

For details see the text. Buffer solutions with the following pH values were used: pH 7.50 ($\bullet$), 7.80 ($\blacksquare$), 8.25 ($\blacktriangle$), 8.5 (Δ) and 8.8 ($\square$). The inset shows a plot of v , the reconstitution velocity extrapolated to $I = 0$, against pH. The reconstitution velocity is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme and coenzyme for 30 s at room temperature ($18 \pm 1^\circ\text{C}$).

ionic strength, independently of pH. Therefore the increase in reconstitution rate with pH observed in the acidic region (Fig. 1) was only apparent, a consequence of increasing ionic strength when passing from pH 4.2 to 5.5.

Substantially the same results were obtained for the reconstitution of aspartate aminotransferase with pyridoxamine phosphate in the acidic region. In the alkaline region the reconstitution rate was a linear function of ionic strength (Fig. 6); the variation of reconstitution rate with pH, obtained by extrapolating to $I = 0$ the linear plot of Fig. 6, is represented by an S-shaped curve that is consistent with that mentioned above (inset, Fig. 6).

Effect of inorganic univalent ions

In order to investigate the effect of NaCl previously used for adjusting the ionic strength of the acetate and Tris buffers, the reconstitution rate of aspartate aminotransferase was measured at pH 5.5 and 8.25 in the presence of NaF, NaCl, NaBr (NaI was not used because of the presence of trace amounts of iodine that readily inactivated the apoenzyme) and of LiCl, KCl, RbCl and CsCl, in concentrations ranging from 0.1 to 1.0 M, except for NaF, whose solubility did not permit preparation of incubation mixtures in which the halide salt concentration exceeded 0.3 M. Unfortunately, the many experiments required by this investigation were necessarily carried out with several apoenzyme preparations obtained from enzyme batches of different specific activity (probably owing to variable amounts of β -form, derived from the α -form on aging), so that a direct comparison of ion effect was not possible even when results were reported on a percentage basis. Further, the accelerating effect of some ions on the reconstitution made it difficult to perform accurate measurements of initial velocities by the manual technique described in the Experimental section. Results reported in Table 1 were obtained in experiments carried out with a single apoenzyme preparation reactivated in the presence of 0.3 M halide salt by only a 2-fold excess of coenzyme. By assuming that the effects of cation and anion are additive, it appears that F^- generally accelerates reconstitution whereas Cl^- and Br^- behave differently according to the form of coenzyme and pH value of the medium.

The reconstitution in the presence of LiCl, KCl, NaCl, RbCl and CsCl seemed to reflect the predominant effect of the anion, although some cationic contribution cannot be excluded. At pH 5.5 the following order of effectiveness was observed in the slight accelerating effect on the reconstitution with pyridoxal phosphate: $\text{NaCl} > \text{KCl} > \text{RbCl} > \text{CsCl}$ (LiCl was ineffective); almost the same order ($\text{NaCl} > \text{KCl} > \text{RbCl} > \text{CsCl} > \text{LiCl}$) was observed in the

Table 1. *Effect of halogen ions on the reconstitution rate of aspartate aminotransferase*

The salt concentration was 0.3 M. Coenzymes were present in a 2-fold excess with respect to apoenzyme. Reconstitution rate is expressed as activity ($\Delta E_{280}/\text{min}$) of the holoenzyme formed on incubation of apoenzyme with pyridoxal phosphate for 7 s or with pyridoxamine phosphate for 30 s respectively. For details see the text. Relative rate values are reported in parentheses.

Salt added to buffer	$10^3 \times$ Reconstitution rate with pyridoxal phosphate at		$10^3 \times$ Reconstitution rate with pyridoxamine phosphate at	
	pH 5.5	pH 8.25	pH 5.5	pH 8.25
None	52 (1.00)	40 (1.00)	19 (1.00)	28 (1.00)
NaF	83 (1.59)	75 (1.87)	31 (1.64)	49 (1.74)
NaCl	69 (1.33)	32 (0.80)	43 (2.26)	36 (1.28)
NaBr	50 (0.96)	34 (0.85)	35 (1.84)	33 (1.18)

marked accelerating effect on the reconstitution with pyridoxamine phosphate. At pH 8.25, where the reconstitution with pyridoxal phosphate is depressed by Cl^- (see Table 1), the following order of effectiveness was observed: $\text{KCl} > \text{NaCl} > \text{LiCl} > \text{RbCl} > \text{CsCl}$; the same order was observed in the accelerating effect on the reconstitution with pyridoxamine phosphate.

Conclusion

Results reported above indicate that the reconstitution of aspartate aminotransferase is a more complicated process than might have been thought before the present investigation. It can only be said that in the acidic region the binding of both pyridoxal phosphate and pyridoxamine phosphate to apoenzyme involves the same functional groups. Any attempt to interpret the reconstitution-rate-pH curves by conventional theoretical treatments such as Dixon's (1953) would leave wide margins for arbitrary conclusions. However, some role seems to be played by the ionic strength and univalent ions in the reconstitution process. In particular, changes in water structure caused by order-producing and order-destroying ions (Choppin & Buijs, 1963), such as those used in the present investigation, may create environmental conditions more or less favourable to the interaction between certain functional groups of apoenzyme and coenzyme involved in the binding.

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