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Part of the phospho group of pyridoxal phosphate may titrate over the pH range 5–8 in aspartate aminotransferase

The ^{31}P n.m.r. spectrum of aspartate aminotransferase (EC 2.6.1.1) shows a pK of about 6.5 (the value varies with isoenzyme and with salt concentration) in the pH-dependence of the chemical shift of the phosphorus atom of the pyridoxal phosphate. When this was first observed, it was attributed to titration between $-\text{PO}_3\text{H}^-$ and $-\text{PO}_3^{2-}$; it was soon realized, however, that the change in shift was too small for this conversion (Mattingly *et al.*, 1982; Korpela *et al.*, 1987a,b; Schnackerz *et al.*, 1989; Sanchez-Ruiz *et al.*, 1991). These three groups of authors therefore concluded (1) that the group was in the $-\text{PO}_3^{2-}$ form throughout the pH range 5–8 [Sanchez-Ruiz & Martinez-Carrion (1986) and Sanchez-Ruiz *et al.* (1991) qualified this by saying merely that it was mainly in the dianionic form], and (2) that an ionization of pK 6.5 elsewhere in the protein was affecting the phosphorus, e.g. by changing O–P–O bond angles.

Although the arguments were convincing that complete titration of $-\text{PO}_3\text{H}^-$ to $-\text{PO}_3^{2-}$ would give a much larger change in chemical shift than that observed, no one argued whether a fraction of the group might not possess the observed pK. Dixon & Tipton (1973) pointed out that, to a close approximation,

Scheme 1. The ionization of the postulated acid

The pK values marked on the scheme are those of the groups, and those to the right are the titration pK values, which are so far apart that they differ negligibly from the molecular values. It may be noted that although the higher of the two group values for the phospho group is 5.8, it is with a pK of 6.5 that a fifth of it titrates; this titration pK is dominated by the value for $-\text{AH}$, since most of this value is contributed by the dissociation of $-\text{AH}$.

ionizing groups, if capable of interacting with others, can titrate in fractions. Dixon *et al.* (1991) recently showed that this statement is exact, and not merely an approximation. Thus a fraction of one group will have one pK, and the rest will have another (or other fractions will have others if the group interacts with more than one other ionizing group). The one H^+ released by the protein with each pK is made up of the fractions from different groups that possess this pK. Hence a small fraction of the phospho group might indeed be in the $-\text{PO}_3\text{H}^-$ form, and change the shift of the phosphorus atom as it titrates to form $-\text{PO}_3^{2-}$.

Fig. 1. Curves of the pH-dependences of the relative concentrations of a dibasic acid

The relative concentrations of the ionic forms of the acid $\text{HA}-\text{PO}_3\text{H}^-$ are shown: (1) $[\text{HA}-\text{PO}_3\text{H}^-]$; (2) $[\text{HA}-\text{PO}_3^{2-}]$; (3) $[\text{A}^--\text{PO}_3\text{H}^-]$; (4) $[\text{A}^--\text{PO}_3^{2-}]$; (5) = (2) + (3), i.e. the total concentration of the singly dehydrated form; (6) = (2) + (4), i.e. the total concentration of the form $-\text{PO}_3^{2-}$; (7) = (3) + (4), i.e. the total concentration of the form $-\text{A}^-$. These curves are obtained by assigning the $-\text{PO}_3\text{H}^-$ group a pK of 2.1 (intersection a) when the other group is in the form $-\text{AH}$, and 5.8 (intersection c) when the other group is in the form $-\text{A}^-$ (i.e. $-\text{AH}$ greatly stabilizes the dianionic form of the phospho group, e.g. by hydrogen bonding, lowering its pK greatly in comparison with a normal phosphate), and assigning the $-\text{AH}$ group a pK of 2.7 (intersection b) when the phospho group is in the $-\text{PO}_3\text{H}^-$ form, and 6.4 (intersection d) when the phospho group is in the $-\text{PO}_3^{2-}$ form, as shown in Scheme 1. The assignment of a difference of 0.6, i.e. $\log_{10}(4)$, between the pK values of the two groups gives a 4:1 ratio between the two monohydrated forms, so that 20% of the group $-\text{PO}_3\text{H}^-$ titrates with one pK, which is 6.5 for the actual values of group constants chosen (curve 6). The remaining 80% of the H^+ released with this titration pK comes from the group $\text{HA}-$; the remaining 80% of the phospho group titrates with the pK of 2.

Fig. 1 and Scheme 1 give conceivable assignments that could lead to the behaviour seen. As all those who have investigated the spectra are agreed, a group $-AH$ greatly stabilizes the $-PO_3^{2-}$ form; the change in group pK postulated here is from 5.8 to 2.1 when $-A^-$ is converted into $-AH$ (the value of the lower pK is comparatively unimportant). These assignments, with a value of 6.4 for $-AH$ when the phospho group is $-PO_3^{2-}$, would lead to a fifth of the phospho group titrating with a pK of 6.5. The same conclusions can be reached without explicit use of the fact that each group has fractions that possess the different titration pK values of the molecule; thus Gorenstein *et al.* (1976) proposed essentially the same model for the interaction of the phospho group of nucleotides with ribonuclease.

No attempt is made to assign actual values from the published data. The change in chemical shift for model compounds in solution as $-PO_3H^-$ is titrated to $-PO_3^{2-}$ depends greatly on the medium (Korpela *et al.*, 1987a,b), so it is not possible to say what fraction of the phospho group might titrate at the observed pK . Indeed, all assignments of group constants are based on assumptions (Dixon, 1976), although these can have a firm rational basis (e.g. Metzler *et al.*, 1973; Gorenstein *et al.*, 1976).

Fig. 1 is not meant to imply that the actual curves shown could be followed by the enzyme in the lower part of the pH range given; denaturation due to hydronation of many carboxylate groups would follow taking the pH so low. The region 0–5 is shown only to illustrate the meaning of the pK values discussed.

This demonstration that the observed shift may be due to titration of part of the phospho group does not mean that this is the true explanation. It is still perfectly conceivable that a nearly ionization changes the environment of the phosphorus atom in some other way. Even if the shift is indeed due to titration of a small fraction of the phospho group, this alters the previous conclusions rather little. The conclusions that the phospho group is dianionic over the whole pH range 5–8 would be valid for most of the group [as Sanchez-Ruiz *et al.* (1991) stated], just as the conclusion that the ionization of pK 6.5 must be elsewhere would be valid for most of that ionization. The conclusion that the $-PO_3^{2-}$ form of the phospho group is stabilized in the enzyme by a peculiar environment would also remain justified. The ratio between the forms $-A-H \cdots O-PO_2^-$ and $-A^- \cdots H-O-PO_2^-$ may merely reflect the relative sojourn times of a rapidly mobile hydron.

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A common pattern between the TGF- β family and glutaredoxin

Transforming growth factors- β (TGF- β 's) constitute a superfamily of polypeptide factors involved in the control of cell growth and differentiation. The superfamily includes a variety of short proteins whose functions, although only partially characterized, are diverse and even antagonistic (Massagué, 1987; Sporn & Roberts, 1990). Three regions of sequence conservation can be identified in the C-terminal domain of the mature TGF- β polypeptides (Wozney *et al.*, 1988; see Fig. 1a). The first region is statistically the most significant. We have found that, with the exception of a large insertion, this region is very similar to a region in animal glutaredoxins (Fig. 1a). We have derived an amino acid class covering pattern (Smith & Smith, 1990) from the sequence of animal glutaredoxins and TGF- β 's in this region (see Fig. 1b) and found its information content equal to 9.14. That is, the similarity implied by this pattern is statistically equivalent to sharing more than nine identical amino acids. Such a degree of similarity between animal glutaredoxin and TGF- β 's is significant, since the overall degree of conservation within TGF- β 's in this region is statistically equivalent to sharing no more than 13 amino acids. In other words, of 13 conserved amino acid equivalences among TGF- β 's in this region, nine of them are also conserved in glutaredoxins. We have observed that a pattern's information content correlates with its functional specificity (Guigó *et al.*, 1991). In general, patterns with information content greater than 8 approach 100% specificity, while those with information content below 6 show little functional specificity. In fact, the pattern is not present in any protein in the SWISS-PROT database, release 13 (EMBL, 1990), other than TGF- β 's and animal glutaredoxins.

Glutaredoxins (or thioltransferases) have been proposed to exert a general thiol redox control on proteins by acting as effective thiol–disulphide oxidoreductases (Holmgren, 1985; Ziegler, 1985), a function they share with thioredoxins. Interestingly, the aforementioned pattern contains the animal glutaredoxin second dithiol site, which is missing from *Escherichia coli* and yeast glutaredoxin. It is still unknown whether this second dithiol site is essential for enzyme activity. However, a similar arrangement of thiols has been observed in thioredoxins, where microbial thioredoxins have only one dithiol, whereas animal thioredoxins contain an additional one (Johnson *et al.*, 1988). The fact that this configuration is not present in bacterial thioredoxin and glutaredoxin, but has been retained in both proteins of animal origin, suggests that the second dithiol may have an important function (Hopper *et al.*, 1989).

Dithiol active groups seem to play a role in hormone–receptor interactions (Robillard & Konings, 1982; Grippo *et al.*, 1985). Glutaredoxin, for example, has been shown to enhance the binding of L-tri-iodothyronine (T3) to its receptor by preserving the T3 receptor in a reduced state (Takagi *et al.*, 1989). Thiol-reducing activity has been recently discovered in the gonadotropin hormones themselves. In this case, a dithiol site in gonadotropins would be responsible for disulphide cross-linking, leading to the formation of an irreversible hormone–receptor complex (Boniface & Reichert, 1990). These observations have suggested the possible existence of an structural motif common to the dithiol sites of thioredoxin, glutaredoxin, disulphide isomerases and gonadotropins (L. Ellis, personal communication).

The mechanism of activation of the TGF- β receptor is still unknown and, as has been pointed out in a recent review (Massagué *et al.*, 1990), attempts to determine whether TGF- β is acting through any of the known signalling pathways (those involving tyrosine protein kinases, G proteins, ion channels or phosphatidylinositol phospholipases/C kinases) have generally