Multiple binding of thallium and rubidium to potassium-activated yeast aldehyde dehydrogenase

Influences on tertiary structure, stability and catalytic activity

Keith A. BOSTIAN* and Graham F. BETTS†

Department of Plant Biology and Microbiology, Queen Mary College, Mile End Road, London E1 4NS, U.K.

and Wing K. MAN‡ and Martin N. HUGHES

Department of Chemistry, Queen Elizabeth College, Campden Hill Road, London W8 7AH, U.K.

(Received 5 April 1982/Accepted 7 July 1982)

Univalent cation activators of aldehyde dehydrogenase have dual effects, both interpreted as cation-induced or -stabilized conformation changes. These two processes are differentiated by the time scales of their associated changes in activity. Using Tl^+ as an activator, under certain conditions, the slower change in activity saturates at a Tl^+ concentration which is only $0.1 K_s$ for the faster change. This, together with evidence for cation-induced rather than cation-stabilized conformation changes, is used to propose separate binding sites for cations responsible for the two activation processes. Equilibrium dialysis indicates 4 binding sites per active site for Rb^+ or 6 sites for Tl^+ . At least one of the additional sites for Tl^+ is an inhibitory site which has been differentiated from the activator sites on the basis of steady-state and pre-steady-state kinetic data.

A number of enzymes show an absolute univalent cation requirement for catalytic activity, and for a majority of these enzymes K^+ is the most effective activator (Evans & Sorger, 1966; Suelter, 1970). We have studied yeast aldehyde dehydrogenase.

It has been shown that the activity of yeast aldehyde dehydrogenase is rapidly lost on removal of activating K^+ (Black, 1951). Provided the activator cation environment is quickly restored enzyme activity rapidly recovers (Springham & Betts, 1973; Betts *et al.*, 1979). However, continued incubation of enzyme in the absence of activator results in the accumulation of an enzyme form attaining maximal activity only after a 10 min recovery in the presence of K^+ (Sorger & Evans, 1966). We have interpreted these enzyme activity changes in terms of a mechanism whereby K^+ induces two conformation transitions (Betts *et al.*, 1979). A model in which K^+ binding at a single site could induce or stabilize either transition independently was eliminated. Two alternatives were offered,

either that the transitions are accomplished by ligand stabilization of an active conformation which alone binds K^+ significantly, or that the fast and slow transitions are promoted by K^+ binding at separate and independent sites (Betts *et al.*, 1979). We now present evidence which is consistent with the second of these alternatives.

Experimental

N.G. and S.F. yeast (British Fermentation Products Ltd., London EC2, U.K.) was obtained from a local baker. Enzyme for most of the kinetic and stability studies was purified to stage III by the procedure of Bostian & Betts (1978a). This fraction contained 4.5–5 mg of protein/ml; 1 mg of protein catalysed the production of 1–3 μ mol of NADH/min in the standard assay below except that 0.1 M-KCl was used as the activating salt. Enzyme was stored in small portions at -18°C in a buffer consisting of 0.025 M- K_2HPO_4 /10 mM-2-mercaptoethanol, adjusted to pH 6.6 with 1 M-citric acid at 4°C . In all cases, enzyme was thawed when required, equilibrated with an appropriate buffer by chromatography on Sephadex G-25 at 4°C , and diluted to a convenient activity. Most buffers, including Sephadex chromatography buffer, contained 0.1 M-Tris/ HNO_3 (pH 8.0)/1 mM-2-mercaptoethanol, and this is

* Present address: Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02254, U.S.A.

† To whom correspondence should be sent.

‡ Present address: Department of Surgery, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0HS, U.K.

referred to throughout as 'Tris buffer'. From the time of Sephadex chromatography the enzyme was kept in Tris buffer with or without additions throughout subsequent procedures. Homogeneous enzyme was prepared by the method of Bostian & Betts (1978a); 1 mg catalysed the production of 34 μmol of NADH/min in the standard assay below except that 0.1 M-KCl was the activating salt.

Enzyme activity was measured by recording the increase in A_{340} due to the production of NADH. Standard assays were conducted with a Pye-Unicam SP. 1800 spectrophotometer, in a reaction mixture of 2.5 ml, containing 0.1 M-Tris/HNO₃ (pH 8.0)/1 mM-2-mercaptoethanol/0.5 mM-NAD⁺/2 mM-acetaldehyde, and various concentrations of KNO₃ or TlNO₃ and enzyme. Enzyme was added last to initiate the reaction. The reaction temperature was 25°C. Rapid-mixing experiments were performed on a Durrum 1300 rapid-mixing and stopped-flow apparatus (Durrum Instruments, Palo Alto, CA, U.S.A.) with a path length in the optical chamber of 2 cm. The contents of the two rapid-mixing syringes were arranged to give final reaction concentrations the same as that described above, except for changes in cation concentrations as detailed in the Figure legends.

Equilibrium dialysis binding experiments were performed according to the published procedure (Bostian & Betts, 1978a), except that ²⁰⁴Tl⁺ or ⁸⁶Rb⁺ were used instead of labelled pyridine nucleotide. Equilibration time with these cations was determined as 90 min. For Rb⁺ binding studies the dialysis buffer was 0.1 M-Tris/HCl (pH 8.0)/20% (v/v) ethanediol/1 mM-2-mercaptoethanol. For Tl⁺ binding experiments Tris/HCl was replaced by Tris/HNO₃.

²⁰⁴Tl⁺ solutions were prepared from ²⁰⁴Tl₂SO₄ (Amersham/Searle; 0.73 Ci/mmol in 0.1 M-H₂SO₄) by diluting with non-radioactive 0.25 M-TlNO₃. After dialysis, three samples of 85 μl were withdrawn and added to 10 ml of 1% 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) in toluene/Triton X-100 (1:1, v/v) by gravimetric procedures, and radioactivity was determined in a Phillips liquid scintillation counter with channel setting of 40–1000 discriminator units, an attenuation of 3.9%, and with external and sample channel ratios. Samples were counted for a total of 2.7×10^6 counts. With weight corrections for sample and scintillant volumes, standard deviation was less than 0.1%.

⁸⁶RbCl solutions were prepared from a 0.2 M stock solution of RbCl containing ⁸⁶RbCl (Amersham/Searle; 1.47 Ci/mmol) at a specific radioactivity of 0.48 mCi/mmol. After dialysis three 60 μl aliquots from each chamber were removed and added to 5 ml of H₂O, gravimetrically, in plastic scintillation vials. Radioactivity was measured in a Packard Auto-

gamma counter. The photopeak was straddled by setting a 10% gain channel from 160–320 discriminator units. Vials were counted three times for 10 min each, giving an accumulated count of at least 10⁵ counts for each sample. The course of counting times was recorded for subsequent corrections for radioactive decay. Weight and decay corrected readings had a standard deviation less than 0.15%.

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Sephadex G-25 was obtained from Pharmacia; Trizma base, NAD⁺ (free acid; grade VI), bovine serum albumin (fraction V) and 2-mercaptoethanol were obtained from Sigma. Acetaldehyde was redistilled and stored at -18°C as a 2 M solution in water. All other chemicals were AnalaR grade, except for reagent grade K₂HPO₄.

Results

Tl⁺-induced changes in activity

Tl⁺ has previously been demonstrated to be an effective activator of aldehyde dehydrogenase, with a dissociation constant (K_s) measured by its effect on steady state reaction rates of 3.4×10^{-3} M (Bostian *et al.*, 1975). However, increases in enzyme activity due to Tl⁺ saturation at the site responsible for activation are offset by an inhibition that is apparent at Tl⁺ concentrations in excess of 10^{-3} M. We have further investigated the nature of this inhibition. This was done by curve-fitting to rate equations for various types of classical inhibition (Laidler & Bunting, 1973). In Fig. 1 the points are experimental data and the lines are best fit assuming that: in curve (a), Tl⁺ is both an activator and a purely non-competitive inhibitor with respect to Tl⁺; curve (b), that Tl⁺ is an activator plus purely uncompetitive inhibitor with respect to Tl⁺; curve (c), that Tl⁺ is an activator plus a mixed inhibitor such that the rate depends on an equation of the form:

$$v = \frac{V_{\text{max.}} [\text{Tl}^+]}{K_s \left(1 + \frac{[\text{Tl}^+]}{K_i} \right) + [\text{Tl}^+] \left(1 + \frac{[\text{Tl}^+]}{K_i} \right)} \quad (1)$$

where substrates are kept constant, K_i and K_1 are written as dissociation constants for the inhibitor and where the restriction that $K_i = K_1$ is removed.

As can be seen from Fig. 1 the best fit is to the mixed inhibition case [$\sum (v^{\text{expt.}} - v^{\text{calc.}})^2 = 0.00615$ compared with 0.0136 for curve (a) and 0.0171 for curve (b)]. The dissociation constants for Tl⁺ binding at the inhibitor sites giving best fit are $K_i = 3.22$ mM and $K_1 = 7.04$ mM. Although it may be that eqn. (1) fails to describe completely the inhibitory pattern of Tl⁺ it seems unlikely that values for dissociation constants vary much from those calculated here.

Fig. 1. Comparison of experimental data for TI^+ activation and inhibition of aldehyde dehydrogenase with theoretical data for classical activation-inhibition mechanisms

A semi-logarithmic plot of the variation of the initial enzyme activity with $\log [\text{TI}^+]$ is shown. Points are experimental data from Bostian *et al.* (1975) in which 0.5 mg of Fraction III enzyme was measured in the standard assay conditions except that TINO_3 was varied as indicated. Lines are: (a), theoretical velocity assuming TI^+ acting as an activator and pure noncompetitive inhibitor with respect to TI^+ with $K_i = 3.8 \times 10^{-3} \text{ M}$; (b), theoretical velocity calculated assuming TI^+ acting as an activator and pure uncompetitive inhibitor with respect to TI^+ with $K_i = 2.6 \times 10^{-3} \text{ M}$; (c), theoretical velocity assuming TI^+ acting as activator and mixed inhibitor (see the text) with $K_{i'} = 3.216 \times 10^{-3} \text{ M}$, $K_i = 7.0 \times 10^{-3} \text{ M}$. Value of $V_{\max} = 1.32 \times 10^{-6} \cdot \text{s}^{-1} \cdot \text{l}^{-1}$; K_s for TI^+ as activator $= 3.4 \times 10^{-3} \text{ M}$.

Additional information on the inhibitory effect of TI^+ at high concentrations was obtained by rapid mixing and stopped-flow experiments. It has been shown that removal of activating K^+ by rapid dilution and addition of Li^+ , which is a competitive inhibitor with respect to activator, gives transiently the high rate of activity expected in the environment existing before the unfavourable change of cation status (Springham & Betts, 1973; Bostian *et al.*, 1975). Activity takes several seconds to reflect the new unfavourable environment. Fig. 2 demonstrates the behaviour when TI^+ concentrations are rapidly changed. This shows that loss of activity due to removal of activator by dilution and competition with non-activating ions (curve a) is different from the inhibition by high concentrations of TI^+ (curve c) and makes it possible that inhibitory TI^+ may be binding at a different site to the activator site.

It is clear from the data of Fig. 2 that burst activity of curve (a) is not due to the step monitored (NADH production) preceeding a rate-limiting step of the normal catalytic cycle, for if this was the case

Fig. 2. Response of aldehyde dehydrogenase to rapid change in cation environment

Fraction III enzyme was equilibrated by chromatography on Sephadex G-25 into 0.1 M-Tris/HNO₃ (pH 8.0)/1 mM-2-mercaptoethanol/3.75 mM-TINO₃ and loaded into one syringe of the rapid mixing and stopped-flow apparatus. The second syringe contained reagents which when rapidly mixed with the contents of the enzyme syringe gave a final reaction mixture of 0.19 mg of enzyme protein per ml, 0.1 M-Tris/HNO₃, pH 8.0, 1 mM-2-mercaptoethanol, 0.5 mM-NAD⁺, 2 mM-acetaldehyde and: curve (a), 1.875 mM TINO₃ and 0.1 M-LiNO₃; curve (b), 3.75 mM-TINO₃; curve (c), 40 mM-TINO₃. Path length in the optical chamber was 2 cm.

a burst should be seen in curve (b). Moreover, as the following calculations show, each enzyme molecule turns over several times to produce the burst of curve (a).

The concentration of aldehyde dehydrogenase in the assays of Fig. 2 is calculated to be $1.6 \times 10^{-7} \text{ M}$ active sites (using a monomer M_r of 62 000; Bostian & Betts, 1978a). The concentration of NADH produced in the burst of Fig. 2, curve (a) $= 3.05 \times 10^{-6} \text{ M}$. Thus $1.6 \times 10^{-7} \text{ M}$ active sites have produced $3.05 \times 10^{-6} \text{ M}$ NADH and therefore must have turned over an average 19 times to give the burst.

TI⁺-induced changes in stability

All of the processes of activation and inhibition dealt with above involve rate changes quite independent of the slow conformation transition (Betts *et al.*, 1979). Results have been presented (Betts *et al.*, 1979) which indicated that the requirement for K^+ in the slow reactivation process is less than that for the rapid reactivation process. This difference is accentuated when TI^+ rather than K^+ is the activator.

Fig. 3 shows the loss of activity when enzyme is incubated at various concentrations of TI^+ prior to assay. Assays were performed at a TI^+ concen-

Fig. 3. *Stability of aldehyde dehydrogenase at various TI^+ concentrations*

Fraction III enzyme was chromatographed on Sephadex G-25 column to give a solution of 3.5 mg of protein/ml in 0.1M-Tris/HNO₃ (pH 8.0)/1mM-2-mercaptoethanol and various TINO₃ concentrations, then incubated at 25°C. Aliquots (0.05 ml) were removed at various times and activity was measured in the standard enzyme assay with 0.1M-KCl immediately (O, Δ, □), or after a further 10 min incubation at 25°C in buffer supplemented to 0.1M-KCl (●, ▲, ■). TINO₃ concentrations during incubation (mM) were: O, ●, 1; Δ, ▲, 4; □, ■, 10.

tration of 4×10^{-3} M, which gives maximal activity. In the sets of data represented by open symbols, samples of enzyme were withdrawn from incubating enzyme and assayed immediately. In the sets represented by closed symbols, samples withdrawn from incubating enzyme were supplemented to a final concentration of 0.1M-KNO₃ and incubated for a further 10 min at 25°C before assay. For those samples preincubated in KNO₃ before assay, any slowly reactivating enzyme which has accumulated during incubation at sub-saturating levels of TI^+ should be fully reactivated before assay (Betts *et al.*, 1979). As can be seen, enzyme subjected to the 10 min 'reactivation' in 0.1M-KNO₃ consistently loses activity faster than enzyme solutions incubated at the same TI^+ concentration and then assayed without the 10 min treatment in 0.1M-KNO₃. There would thus seem to be no evidence that when enzyme is incubated in TI^+ concentrations as low as 1mM any slowly reactivating enzyme species accumulates. Thus one must presume that 1mM- TI^+ is sufficient to saturate for this process. This compares with a K_s for TI^+ in the fast reactivation process of 3.4 mM (Bostian *et al.*, 1975).

To confirm that such low levels of TI^+ are sufficient to satisfy fully the requirement for the slow transition, enzyme was equilibrated against buffer containing various concentrations of TI^+ below 1mM. Each sample was then allowed to incubate for

Fig. 4. *Initial velocity of aldehyde dehydrogenase following incubation at various TI^+ concentrations*

Fraction III enzyme was chromatographed on a Sephadex G-25 column to give a solution of 3.5 mg of protein/ml in 0.1M-Tris/HCl (pH 8.0)/1mM-2-mercaptoethanol and TINO₃ concentrations of (mM): (a), 0.5; (b), 0.3; (c), 0.2; (d), 0.1; (e), 0.05. Enzyme was then incubated at 25°C for 15 min after which 0.05 ml aliquots were removed and assayed in the standard mixture.

15 min at 25°C prior to assay in a reaction mixture containing K⁺ at a concentration optimum for activity. Fig. 4 shows that when enzyme is pre-incubated in as little as 0.5mM- TI^+ there is no evidence of a slow recovery of activity in the reaction mixture. Only at TI^+ preincubation concentrations lower than 0.5mM is there an acceleration of rate during the assay period, indicating the reactivation of slowly reactivating forms which have accumulated during the preincubation period. This suggests a binding site for TI^+ in this interaction with a $K_s \ll 1$ mM.

The ability of TI^+ to reactivate these slowly reactivating forms is shown in Fig. 5. Enzyme was first depleted of activating cations by gel chromatography, then incubated for 15 min at 25°C, by which time initial velocity had decayed to near zero. Incubating enzyme was then supplemented to various concentrations of TI^+ , aliquots were removed at various times and assayed in the standard reaction mixture, except that the activating cation was 4mM-TINO₃. The rate and the maximum levels of reactivation increased as the TI^+ concentration was increased up to 40mM. Further increase in TI^+ concentration resulted in appreciable protein precipitation and lower levels of reactivation. However, as seen from the data, the concentration of TI^+ required for reactivation is markedly higher than that required to prevent the

Fig. 5. Reactivation of aldehyde dehydrogenase by Tl^+ following incubation in Tris buffer

Fraction III was chromatographed on Sephadex G-25 columns to give a solution of 4.9 mg of protein/ml in 0.1 M-Tris/ HNO_3 (pH 8.0)/1 mM-2-mercaptoethanol. After incubation at 25°C for 15 min, enzyme was supplemented to various TlNO_3 concentrations and 0.05 ml aliquots were removed at various times and assayed for initial enzyme activity in the standard reaction mixture except that the cation activator was 4 mM- TlNO_3 . The figure shows a plot of variation of initial activity of the above treated enzyme with time after supplementation to concentrations of TlNO_3 (mM): ●, 1; △, 4; ■, 10; ▲, 25; □, 40; ○, 50.

Fig. 6. Binding of Tl^+ to aldehyde dehydrogenase measured by equilibrium dialysis

Homogeneous enzyme was equilibrated by passage through a Sephadex G-25 column with 0.1 M-Tris/ HNO_3 (pH 8.0)/1 mM-2-mercaptoethanol/20% (v/v) ethanediol to give a final enzyme concentration of approx. 8.25 μM . Enzyme solution (0.3 ml) was placed in one half of a dialysis cell and dialysed as described in the text against 0.3 ml of the same buffer but minus enzyme and containing various concentration of $^{204}\text{TlNO}_3$. A Scatchard plot of the binding data is shown.

slow accumulation of a slowly reactivating enzyme form (Figs. 3 and 4).

Univalent cation binding to aldehyde dehydrogenase

Direct binding of inorganic univalent cations to proteins has been studied previously, for example with Na^+/K^+ -ATPase (Matsui *et al.*, 1977).

Equilibrium dialysis experiments were performed by the procedure previously used to measure pyridine nucleotide binding to aldehyde dehydrogenase (Bostian & Betts, 1978a). Molarity of enzyme was based on an M_r of 248 000 (Bostian 1978a). The concentration of ligand was varied from a fractional saturation of 0.2 to 0.8. Data are presented as Scatchard plots in Fig. 6 for Tl^+ and in Fig. 7 for Rb^+ . Rb^+ appears to be very similar to K^+ in this system (Black, 1951) but has an isotope of more convenient half-life. Both Scatchard plots demonstrate multiple independent binding sites having the same intrinsic association constant. The

Fig. 7. Binding of Rb^+ to aldehyde dehydrogenase measured by equilibrium dialysis

The procedure was as in Fig. 6 except that Tris/HCl replaced Tris/ HNO_3 and $^{86}\text{RbCl}$ replaced TlNO_3 . A Scatchard plot of the binding data is shown.

number of binding sites for cation per protein molecule and their association constant were determined from slopes and intercepts of these plots. For Tl^+ there are a total of 23 binding sites per protein molecule with a dissociation constant (K_s) of $3.1 \times 10^{-3} \text{ M}$. For Rb^+ there are a total of 16 binding sites per protein molecule with a K_s of $6.7 \times 10^{-3} \text{ M}$.

Discussion

It has previously been shown (Betts *et al.*, 1979) that K^+ , as well as inducing or stabilizing a rapid activation process complete within a few seconds, also induces or stabilizes a slower activation process complete within 10 min. Both processes are likely to involve a conformational change which can be represented by a thermodynamic square such as that shown below. Presented is the rapid transition (transition II), in the nomenclature of Betts *et al.* (1979).

where $_{\text{II}}$ represents a conformation transition which occurs rapidly and $_1$ represents a conformation transition which occurs slowly. ${}_1\text{E}_{\text{II}}$ is the only active conformation and is active whether or not K^+ is bound. ${}_1\text{E}$ and K_1E are non-active but may rapidly reactivate via the pathways represented by K_6 and K_5 . K_4 and K_7 are written as dissociation constants, $k_{\text{off}}/k_{\text{on}}$. Activity in the absence of K^+ is less than $V_{\text{max}}/100$ so $K_6 > 100$. As it is unlikely that a substantial proportion of enzyme remains in the inactive form in the presence of saturating concentrations of activator then a value $K_5 < 0.1$ may be suggested. To satisfy thermodynamic requirements:

$$K_4 K_5 = K_6 K_7$$

and thus $K_4 > 1000 K_7$.

But $K_4 K_5 = K_6 K_7 = K_s$ (Betts *et al.*, 1979) for the univalent cation involved in this fast conformation change, and $K_s = 7.5 \text{ mM}$ with KCl (Betts *et al.*, 1979). Substituting these values in the equation describing K_s then:

$$\begin{aligned}
 7.5 &= 0.1 K_4 \\
 K_4 &= 75 \text{ mM}
 \end{aligned}$$

In the absence of K^+ the rapidly reactivating form ${}_1\text{E}$ will further transform to a species which only slowly reactivates on returning to K^+ . But ${}_1\text{E}$ binds K^+ only loosely ($K_4 = 75 \text{ mM}$) and, by argument identical to that above, K^+ binding to the form which

has lost state I (that is, form E) will be negligible at concentrations of K^+ known to saturate for activity. To overcome this difficulty two alternatives were offered (Betts *et al.*, 1979).

The first was that activation of E, the slowly reactivating form, is accomplished by K^+ binding at a single site on a small pool of ${}_1\text{E}_{\text{II}}$ existing in the absence of activator and stabilizing it, thereby displacing the equilibrium shown below from left to right.

Two consequences of such a scheme follow. The first is that in order to accomplish the slow reactivation step the fast must also be accomplished. In this case the overall K^+ requirement for the transition from E to ${}_1\text{E}_{\text{II}}$ will be larger than for the transition from ${}_1\text{E}$ to ${}_1\text{E}_{\text{II}}$ by an amount equal to the ratio $\text{E}/{}_1\text{E}$ at equilibrium in the absence of K^+ . Neither of these predictions are supported by the data presented here. Thus Figs. 3 and 4 show that 1 mM-Tl^+ is sufficient to prevent completely the accumulation of E yet 3.4 mM-Tl^+ is required to half-saturate for the rapid activation, that is, the transformation shown below (Bostian *et al.*, 1975).

The second consequence of the scheme for a single activating ion is that it is a scheme of ion-stabilized rather than ion-induced conformation change. But Fig. 5 shows that rates of reactivation of the slow reactivation process increase with increasing cation concentration rather than decrease to a minimum value. This is consistent with a conformation change being cation-induced rather than cation-stabilized (Gutfreund, 1972).

Also consistent with a cation-induced conformation change, this time for the rapid reactivation phenomenon, are differences in substrate saturation characteristics between the enzyme in Tl^+ and that in K^+ (data not shown). If cation activator was stabilizing an active conformation already present at low concentrations in the absence of activator then the same form, with the same kinetic characteristics, should be stabilized no matter what stabilizing cation is used.

The above considerations, which argue against a process of cation stabilization of the active form by cation binding at a single type of site, still leave the alternative offered by Betts *et al.* (1979) that the rapid and slow reactivation process may be induced by activating-cation binding at separate sites; that is, not one but two activating ions are required to maintain each independent active site in its correct geometry.

Data from direct binding studies is presented in

Figs. 6 and 7. If each identical subunit of tetrameric aldehyde dehydrogenase (Clark & Jakoby, 1970) required two univalent cations for activation this would imply a total of eight cation binding sites. The data for Rb⁺ binding provides 16 binding sites. Tl⁺ binding data gives a best fit to 23 binding sites, which may be interpreted as 24 sites as the nearest multiple of 4. In view of Tl⁺ inhibition, which appears from Figs. 1 and 2 to be at a separate site from activation, additional Tl⁺ binding sites were to be expected. It is worthy of note that K_s for Tl⁺ in activation of the fast step (3.4 mM; Bostian *et al.*, 1975), K_s for Tl⁺ in equilibrium dialysis experiments (3.1 mM) (Fig. 6) and K_i giving best fit for Tl⁺ inhibition data (legend of Fig. 1) are comparable.

It would be of interest if these estimates of Tl⁺ binding affinity could be compared with estimates of Tl⁺ binding affinity at the site responsible for the slow reactivation process. Tl⁺ requirement for the slow activation cannot be determined from the asymptotic values of reactivation at various Tl⁺ concentrations shown in Fig. 5. This is because at subsaturating concentrations of activator, various conformers will exist, some of which decay to an irreversibly inactivated state at rates comparable to the rate of the slow reactivation process (Betts *et al.*, 1979). Thus true equilibrium between all potentially active forms is not reached and the fraction of molecules which have achieved the slow reactivation step may be under-represented when compared with an equilibrium limited to all potentially active species.

In spite of the above considerations there seems no doubt that concentrations of Tl⁺ required both for reactivation of the slowly reactivating form (Fig. 5) and to saturate the sites measuring by equilibrium dialysis (Fig. 6) are hugely in excess of those required to prevent the decay of enzyme to the slowly reactivating form as indicated by Figs. 3 and 4. An attempt to resolve this apparent inconsistency is made as follows.

A number of considerations would suggest that the character of the slow conformation change is susceptible to facile alteration. Thus Betts *et al.* (1979) reported that in an occasional crude preparation, NAD⁺ was able to promote the slow reactivation in place of K⁺. Similarly, with homogeneous preparations in the presence of 25% (v/v) ethanediol, K⁺ could no longer promote the slow reactivation but NAD⁺ could (Bostian & Betts, 1978b). In the absence of ethanediol K⁺ could still accomplish the slow reactivation process. It is to be noticed that prior to the reactivation in the experiment of Fig. 5, enzyme has been incubated for 15 min at 25°C in the absence of activator. It is feasible that enzyme which has survived this denaturing environment, even when reactivated, is not identical with enzyme which has not been so

treated. Similarly homogeneous enzyme used for equilibrium dialysis in the presence of 25% ethanediol and incubated for 90 min in only low activator concentrations may no longer demonstrate univalent cation activator binding sites of high affinity at a site responsible for the slow reactivation.

Throughout all the treatments mentioned in the last paragraph, K_s for K⁺ in the fast reactivation process remains remarkably constant, another indication that this is a process independent of the slow cation-dependent activation process.

It would have been more satisfying for the model proposed here if equilibrium dialysis data pointed to 8 binding sites for Rb (two sites for each monomer of tetrameric enzyme) and at least 12 for Tl⁺ (8 plus at least one additional inhibitor site per monomer). Both values obtained from Figs. 6 and 7 are clearly higher than these.

Multiplicity of univalent cation binding sites for which there is no functionally known significance has been established for several proteins. For creatine kinase at pH 9.0, 9 K⁺ ions are bound per enzyme molecule (Floyd & Frieberg, 1966). From a study at its isoelectric point a maximum of 11.6 binding sites exist with a dissociation constant of 6.25×10^{-2} M. There is no evidence to suggest that any of these sites have any kinetic significance. The binding of Na⁺ and K⁺ to myosin and β -lactalbumin has been interpreted by Baker & Saroff (1965) in terms of a mechanism involving chelate formation between univalent cation and protein carboxyl and imidazole or amino groups. The latter protein, of M_r 35000 at pH 9.48, binds up to 4.3 mol of Na⁺ per mol with dissociation constants of 6.6×10^{-3} M.

Thus, multiple 'nonfunctional' univalent cation binding sites with affinity comparable to those in yeast aldehyde dehydrogenase do exist in several proteins. Since there is no *a priori* reason, nor experimental evidence, for more than one K⁺ ion involved in each transition of yeast aldehyde dehydrogenase, it seems reasonable to propose that additional sites under physiological conditions are unimportant both to catalytic activity and to stability of the enzyme. The alternative seems unlikely since in several cases nonhyperbolic kinetics with respect to K⁺ would be observed. The significant result with respect to the question raised in this paper is that binding data provide for sufficient sites to allow a model of a double K⁺ requirement for each independent active site whereas a number of binding sites less than 8 would have eliminated the possibility.

References

- Baker, H. P. & Saroff, H. A. (1965) *Biochemistry* **4**, 1670–1677
- Betts, G. F., Poole, P. L., Springham, M. G. & Bostian, K. A. (1979) *Biochem. J.* **183**, 633–646

- Black, S. (1951) *Arch. Biochem. Biophys.* **34**, 86–97
- Bostian, K. A. & Betts, G. F. (1978a) *Biochem. J.* **173**, 773–786
- Bostian, K. A. & Betts, G. F. (1978b) *Biochem. J.* **173**, 787–798
- Bostian, K. A., Betts, G. F., Man, W. K. & Hughes, M. N. (1975) *FEBS Lett.* **59**, 88–91
- Clark, J. F. & Jakoby, W. B. (1970) *J. Biol. Chem.* **245**, 6072–6077
- Evans, H. J. & Sorger, G. J. (1966) *Annu. Rev. Plant Physiol.* **17**, 47–76
- Floyd, B. F. & Frieberg, F. (1966) *J. Biol. Chem.* **241**, 5533–5536
- Gutfreund, H. (1972) *Enzymes: Physical Principles*, p. 127, Wiley and Sons, London
- Laidler, K. J. & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, pp. 116–119, Clarendon Press, Oxford
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Matsui, H., Hayashi, Y., Homereda, H. & Kimimura, M. (1977) *Biochem. Biophys. Res. Commun.* **75**, 373–380
- Sorger, G. J. & Evans, H. J. (1966) *Biochim. Biophys. Acta* **118**, 1–8
- Springham, M. G. & Betts, G. F. (1973) *Biochim. Biophys. Acta* **309**, 233–236
- Suelter, C. H. (1970) *Science* **168**, 789–795