Kinetic and Spectroscopic Evidence of Cation-Induced Conformation Changes in Yeast K^+ -Activated Aldehyde Dehydrogenase

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The activity, stability and spectroscopic properties of yeast K^+ -activated aldehyde dehydrogenase were measured at various times after removal from, and after returning to a solution containing K^+ . Enzyme activity is rapidly lost on removal of most of the K^+ and rapidly regained if K^+ is replaced immediately. These activity changes are slower than likely rates of K^+ dissociation and association. These rapid changes in concentration result in altered enzyme stability with enzyme in K^+ the more stable. U.v. difference spectra are produced whenever enzyme in an activating environment (K^+ or Tl^+) is compared with enzyme in a non-activating environment ($Tris^+$ or Li^+). These spectral changes occur within 10s. The saturation characteristics with K^+ are hyperbolic for all three phenomena of activation, stabilization and spectral change, with estimated apparent dissociation constants (K_s) for K^+ of 7.5 mM, 5.5 mM and 6 mM respectively. Continued incubation of enzyme in the absence of K^+ results in the accumulation of an enzyme form that re-activates only slowly on replacing K^+ . Stability characteristics in various concentrations of K^+ over equivalent time scales are consistent with the existence of additional conformations. Spectroscopic evidence also indicates such additional slow conformation changes. Results have been interpreted in terms of two separate conformation transitions induced or stabilized by K^+ .

Several enzymes show an absolute requirement for a univalent cation. The majority of these enzymes show maximum activity in the presence of K^+ , whereas activity is often zero in the presence of Li^+ or $Tris^+$ (Evans & Sorger, 1966; Suelter, 1970).

Two general theories of the mechanism of K^+ -activation have been proposed. K^+ is thought to bind either at the active site, where it assists in substrate binding or catalysis (Suelter, 1970), or at a distance from the active site, from where it affects activity by inducing an active conformation (Evans & Sorger, 1966). A prediction of the latter model would be that the proposed protein conformation changes and consequent changes of activity could occur with rate constants markedly different from the rates of binding or dissociation of the activating ion. We present here evidence of cation-induced changes of activated yeast aldehyde dehydrogenase [aldehyde-NAD(P)⁺ oxidoreductase; EC 1.2.1.5] occurring with two different rate constants, both of which are much smaller than the likely rates of cation binding or release.

Stability studies show that enzyme activity decays

at various rates depending upon cation status and pretreatment. The data are entirely consistent with the presence of different proportions of one active and more than one non-active conformation exhibiting various stabilities. Direct evidence of such protein-conformation changes has been sought from investigations of the u.v.-absorption spectra of aldehyde dehydrogenase in various cation environments. We have sought to correlate such evidence with the kinetic evidence on the basis of three criteria. Firstly, do the same ions cause the two effects; secondly, do both phenomena have the same concentration-dependence; and thirdly, do both effects occur at the same rate after a change in cation environment?

U.v. difference spectroscopy has previously been used to investigate conformational alterations of enzymes induced by univalent cation salts. With rabbit muscle pyruvate kinase the ability of an ion to activate does not correlate with the ability to establish the same u.v.-absorption spectrum at physiological concentrations of ions (Suelter *et al.*, 1966; Wilson *et al.*, 1967; Nowak, 1976). Aspartate kinase I from *Escherichia coli* shows u.v.-absorption changes clearly related to activity, but here K^+ is not an essential ion but rather modifies the enzyme behaviour towards substrate, ATP, or feedback inhibitor,

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threonine (Cohen, 1969; Truffa-Bachi *et al.*, 1974). Enolase shows similar u.v. difference spectra (Brewer, 1969), but K^+ is not in this case an activator, and high concentrations of KCl cause dissociation and inhibition of enzyme activity (Gawronski & Westhead, 1969).

Materials and Methods

N.G. and S.F. yeast (British Fermentation Products Ltd., London E.C.2, U.K.) was obtained from a local baker. For most kinetic and stability studies enzyme was purified by heat precipitation and acid precipitation up to fractionation stage II of the purification procedure of Bostian & Betts (1978). This fraction contained between 4.5 and 5 mg of protein/ml and 1 mg of protein catalysed the production of between 1 and 3 μ mol of NADH/min in the standard assay below. Enzyme was stored in small portions at -18°C in a buffer consisting of 0.025 M- K_2HPO_4 /10 mM-mercaptoethanol adjusted to pH 6.6 with 1 M-citric acid at 4°C .

Enzyme preparation was thawed as required and equilibrated with appropriate buffer by chromatography on Sephadex G-25 at 4°C . Eluted enzyme was diluted with the chromatography buffer to give convenient activity. In all cases this buffer included 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol, and this is referred to throughout as 'Tris buffer'. From the time of Sephadex chromatography the enzyme was kept in Tris buffer with or without additions throughout all subsequent procedures.

Homogeneous enzyme was prepared by the method of Bostian & Betts (1978), and 1 mg catalysed the production of 34 μ mol of NADH/min. For spectroscopic studies, homogeneous aldehyde dehydrogenase was equilibrated for use into an appropriate solution by passage through a Sephadex G-25 column at 4°C , and then filtered through a small Millipore-filter assembly attached to a 5 ml disposable syringe.

Enzyme activity was measured by recording the increase in A_{340} due to the production of NADH. Some assays were conducted with a Unicam SP. 1800 spectrophotometer, in which case a reaction mixture of 2.5 ml contained: 0.1 M-Tris/HCl, pH 8.0, 1 mM-mercaptoethanol, 0.5 mM-NAD $^+$, 2 mM-acetaldehyde, various concentrations of KCl, and enzyme. Enzyme was added last to initiate the reaction. The reaction temperature was 25°C . Rapid-mixing experiments were carried out by using a Durrum 13000 rapid-mixing and stopped-flow apparatus (Durrum Instruments, Palo Alto, CA, U.S.A.), in which case contents of the two syringes were arranged to give final reaction concentrations the same as described above, except for changes in cation concentrations detailed in legends to Figures.

Protein difference spectra were obtained by using a Cary 15 spectrophotometer in the autoslit mode with

gain settings such that the maximum spectral bandwidth was less than 0.5 nm. Short pen periods were used, and scans were performed at a speed of 0.5 nm/s. Absorbances were monitored at a scale expansion of 0.1 absorbance unit and all experiments were performed at ambient temperatures. Semimicro cells of 1 cm path length, made of Spectrosil quartz, were selected for use by matching at 280 nm with a 1 mg/ml solution of bovine serum albumin. After electronic adjustment, the baseline did not deviate more than 0.01 Δ unit. Enzyme samples of 1 ml were carefully pipetted with the same bulb pipette into both cells, and baseline scans were made in this 'unperturbed' state. Cells were then removed for addition of small volumes of perturbant or buffer (10–30 μ l), by using an automatic dispensing micro-pipette. The contents were gently mixed by inversion five times and returned to their original position in the spectrophotometer. Either multiple scans were made with time, or after scanning the cells were removed for other additions. Finally an attempt to restore the baseline was made by additions to the reference cell identical with those used previously to perturb the sample cell. At the same time, appropriate dilutions of the sample cell were made. The time required between scans when mixing was performed was usually 2 min, plus 2–3 min for each scan. The last scan recorded in most experiments occurred within 10 min from chromatography, except in experiments where enzyme was purposely allowed to decay further. Residual enzyme activity was determined by the assay above. Except for experiments using nitrate, similar runs without protein resulted in no detectable difference in absorbance in the wavelength region utilized.

Best estimates for the K_s for K^+ inducing activity, stability and spectral shifts were made by using the procedures of Bliss & James (1966). Protein was determined by the biuret method (Gornall *et al.*, 1949) by using bovine serum albumin (Sigma fraction V) as a standard. Sephadex G-25 was obtained from Pharmacia (G.B.), London W.5, U.K. Trizma base, NAD $^+$ (free acid; grade IV), and mercaptoethanol were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. All other chemicals were AnalaR grade, except K_2HPO_4 , which was Reagent grade.

Results and Discussion

Cation-induced changes in activity

Although there is no method available whereby K^+ can be instantaneously removed from a protein solution, a rapid lowering of the effective K^+ concentration can be achieved by diluting out the K^+ in the enzyme sample in one syringe of a stopped-flow apparatus with the contents of the second syringe, which contains substrates and a large concentration

of Li^+ , which is competitive with K^+ (Sorger & Evans, 1966). Fig. 1 (curve A) shows the adjustment of enzyme activity to such a change in cation environment. The rate constant for the decay of active to inactive enzyme is 0.7s^{-1} .

Fig. 1 (curve B) shows that when K^+ is removed from the enzyme by Sephadex chromatography and assayed as quickly as possible, activity recovers in the reaction mixture with a rate constant of 0.4s^{-1} . Curve C shows that the component of the reaction mixture that causes re-activation is K^+ . Here the K^+ was added to the enzyme syringe before the contents were mixed with the contents of the substrate syringe.

The rate constants for the changes in activity consequent on these changes in cation environment are several orders of magnitude slower than the likely rates with which K^+ dissociates from or associates with the enzyme (Williams, 1970; Winkler, 1972). Thus these data are consistent with activity being affected by a relatively slow conformation change in the enzyme induced by the rapid change in cation environment.

The K^+ requirement for the activation process of Fig. 1 is demonstrated in Fig. 2. Activity was measured by conventional spectrophotometry, where

Fig. 1. Response of aldehyde dehydrogenase to changes in cation environment after rapid mixing and stopped flow. The enzyme was equilibrated by Sephadex G-25 chromatography at 4°C with 100mM-Tris/HCl (pH 8.0)/1mM-mercaptoethanol and: curve A, 50mM-KCl; curve B, no KCl; curve C, 100mM-KCl. The contents of the enzyme syringe were mixed with those of the substrate syringe to give reaction concentrations of 100mM-Tris/HCl (pH 8.0)/1mM-mercaptoethanol/2mM-acetaldehyde/0.5mM-NAD $^+$ and: curve A, 25mM-KCl/100mM-LiCl; curve B, 50mM-KCl; curve C, 100mM-KCl. The protein concentration in the final reaction mixture was 0.2mg/ml.

the activation has been accomplished in the 10s between adding enzyme to the reaction mixture and starting the recording. Saturation was hyperbolic, with an apparent dissociation constant (K_s) for K^+ for the set of data shown in Fig. 2 of 7.4mM and a mean value for eleven such determinations of 7.5mM.

In Fig. 3, line '0' is identical with curve B of Fig. 1, except that it was obtained with conventional spectrophotometry and a lower protein concentration was used. Therefore the time scale is smaller and the rapid activation process occurred during the mixing time. Other curves in Fig. 3 were obtained by incubating the enzyme in Tris buffer at 25°C for the times indicated. The longer the enzyme is incubated in Tris buffer the more pronounced is a re-activation process seen to be taking place in the reaction mixtures of Fig. 3. Thus the enzyme no longer re-activates rapidly and appears to decay in part to an enzyme form that recovers activity only over the much longer periods of these assays. Part of the loss of activity on incubation in Tris buffer is irrecoverable, i.e. the rate after several minutes incubation never recovers to the rate before incubation. The initial velocity of each curve of Fig. 3 is a measure of the rapidly re-activating form that survives the continuing incubation in Tris. During the early part of the incubation in Tris the initial velocity decays as a first-order process with a rate constant of 0.18min^{-1} .

The rate constant for the re-activation process occurring in the reaction mixtures of Fig. 3 is obtained from the reciprocal of the intercept of the steepest part of the 30min curve (off the Figure) extrapolated

Fig. 2. Activation of aldehyde dehydrogenase by K^+ . The assay mixtures contained in 2.5ml: 100mM-Tris/HCl, pH 8.0, 1mM-mercaptoethanol, 2mM-acetaldehyde, 0.5mM-NAD $^+$, protein (50 μg) and KCl (as shown in the Figure). The reaction temperature was 25°C .

Fig. 3. Decrease of initial velocity during incubation of enzyme in the absence of univalent-cation activator. The enzyme was equilibrated by Sephadex G-25 chromatography with 100mM-Tris/HCl (pH 8.0)/1mM-mercaptoethanol and incubated at 25°C for the periods of time shown against the curves before assay by conventional spectrophotometry. The reaction mixtures were as for Fig. 1, curve C, except that protein concentration was 30 μ g in a total reaction mixture of 2.5 ml.

back to the time axis. Re-activation occurs with a rate constant of 1.3 min⁻¹.

A component of the reaction mixture of Fig. 3 capable of re-activating the enzyme is K⁺. This is shown in Fig. 4, where the slowly re-activating enzyme form was prepared by incubation of enzyme in Tris buffer until the initial velocity was essentially zero when assayed in reaction mixture containing K⁺. This enzyme was then supplemented with KCl to 100mM, and portions were removed for assay at various times. Re-activation had a first-order rate constant of 0.76 min⁻¹. (The re-activation was essentially complete inside 10min, and this decided the length of the re-activation period used in Figs. 5 and 6 and Table 1 below.)

There are other reports of K⁺-activated enzymes that recover activity only slowly on returning to potassium salts after prolonged dialysis against water of non-activation ions (Green, 1964; Sorger & Evans, 1966; Wilson *et al.*, 1967; Wampler & Westhead, 1968; Bothwell & Datta, 1971).

We have considered whether the difference seen in the rate with which the slow re-activation occurs in the reaction mixture (1.3 min⁻¹ from Fig. 3) and in Tris buffer plus 100mM-KCl (0.76 min⁻¹ from Fig. 4) may be due to substrates contributing to the re-activation process in the reaction mixture. In the usual preparations reported here, neither aldehyde nor NAD⁺ causes the slow re-activation. (Homogeneous

Fig. 4. Re-activation of the slow-re-activating form of aldehyde dehydrogenase by K⁺

The enzyme was equilibrated on Sephadex G-25 with 100mM-Tris/HCl (pH 8.0)/1mM-mercaptoethanol and incubated at 25°C for 1 h. The enzyme was supplemented with an equal volume of 100mM-Tris/HCl (pH 8.0)/1mM-mercaptoethanol/200mM-K⁺, and portions were removed for assay at various times. The reaction concentrations were as in Fig. 1, curve C, except for protein, which was at 130 μ g per assay volume of 2.5 ml. The velocity when $t \rightarrow \infty$ was taken as a measure of the amount of the slow-recovery form present at the start of the re-activation. The amount remaining at various times then = $v_{\infty} - v_t$.

enzyme preparations differ in this respect, since NAD⁺ can cause the slow re-activation; see below.) Neither substrate added separately has any effect on the rate of re-activation caused by K⁺.

The difference between the two estimates of the rate for the slow recovery process (see Figs. 3 and 4) is due at least in part to an error introduced into the estimate of re-activation rate in the reaction mixture by underestimating velocity when $t \rightarrow \infty$. There are factors present that tend to slow the reaction rate. Some of these, such as substrate depletion and product inhibition, can be quantified and corrected for. However, the enzyme appears to be less stable when working in a full reaction mixture than in a similar environment, except for the omission of any one essential component (substrate, K⁺). The amount of

such decay in a reaction that has an opposed re-activation process that one is attempting to quantify is difficult to predict, and no correction for it was introduced into the estimate of the rate of re-activation in the reaction mixture.

We propose that the cation-induced changes in activity demonstrated in Figs. 1–4 involve two conformation transitions. If the conformation transitions called I and II occur independently of each other, then four conformation states can exist, namely E, ${}_1E$, E_{II} and ${}_1E_{II}$.

The rapid-mixing experiments of Fig. 1 demonstrate the loss or gain of conformation state II. That is, conformation state II is rapidly lost by dilution of K^+ and addition of Li^+ or on removal of K^+ by Sephadex chromatography, in which case an enzyme emerges from the column still possessing state I (that is, ${}_1E$). Form ${}_1E$ is rapidly re-activated by the addition of K^+ . However, continued incubation in the absence of K^+ (Fig. 3) results in a slower loss of state I. The resulting conformation, E, can be slowly re-activated to form ${}_1E_{II}$ by the reintroduction of K^+ (Figs. 3 and 4).

Cation-induced changes in stability

Variation in conformation induced by protein ligands often results in altered protein stability. If the various conformations proposed above varied in stability, one would expect complicated decay characteristics depending on the proportion of the various conformations. This in turn would depend on saturation characteristics of the cation-binding site, the prevailing cation concentration and the time elapsed since any change in cation status.

The complications may be eliminated by selecting parameters at extremes, e.g. K^+ and/or time tending towards 0 or ∞ . Thus decay of total recoverable enzyme activity was monitored immediately ($t = 0$) after transferring an enzyme from saturating K^+ to various subsaturating concentrations. The predictions of the model are as follows. Stored in saturating K^+ , both conformations I and II will be maximally induced. Immediately on removal of K^+ on Sephadex columns at $4^\circ C$, conformation state II will have been lost but state I will have survived. If there is a difference in stability between ${}_1E$ (inactive) and ${}_1E_{II}$ (active) forms, then during the early stages of decay, while state I substantially survives, the decay rate will be decided simply by the proportions of enzyme existing as forms ${}_1E$ and ${}_1E_{II}$. Fig. 5 and inset show that the induction of a stable species is a hyperbolic function of K^+ concentration with a K_s for this set of data of 5.6 mM. The mean value for three such determinations is 5.5 mM, and is not significantly different ($P = 0.2$) from the K_s for K^+ -inducing activity.

At subsaturating concentrations of K^+ , rapidly re-activating form ${}_1E$ decays to slowly re-activating

Fig. 5. Effect of K^+ on the short-term stability of aldehyde dehydrogenase

The enzyme was equilibrated on Sephadex G-25 columns at $4^\circ C$ with 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol and KCl at the following concentrations: ○, zero; △, 1.5; □, 3; ●, 7; ▲, 15; ■, 30; ▽, 100 mM. The enzyme was rapidly heated to and incubated at $25^\circ C$. Portions were removed at the times indicated and supplemented with equal volume of 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/200 mM-KCl. Each portion was incubated for a further 10 min at $25^\circ C$, then assayed as in Fig. 1, curve C, except that the protein concentration was approx. $130 \mu\text{g}/2.5 \text{ ml}$ total reaction volume. The inset shows initial slopes of decay curves interpreted in terms of fraction of total enzyme decaying with a rate constant of $k = 0.0084 \text{ min}^{-1}$ (100 mM- K^+ curve), with the remainder decaying with a rate constant of $k = 0.053 \text{ min}^{-1}$ (zero- K^+ curve). ($\bar{Y}$ is the fractional saturation of binding sites; see the text.)

form E. The model will predict that the linearity of the semilogarithmic plot of decay of the irreversibly inactivated conformer, X, seen in Fig. 5 will only be maintained if there is no difference in stability between ${}_1E$ and E forms. Fig. 6 shows the effect of K^+ on stability over periods where significant concentrations of the slow-re-activating form, E, can accumulate. As is seen, the decay is complicated. Deviation from first-order rate might indicate multiple-enzyme forms that are covalently different. Consistent with this is the report of multiple forms of this enzyme produced by the action of endogenous proteinases during extraction (Clark & Jakoby, 1970a,b). However, enzymes prepared in the presence of phenylmethanesulphonyl fluoride, which results in a single active band of enzyme on polyacrylamide-gel

electrophoresis, exhibit the same stability patterns as those shown in Fig. 6. We interpret these decay patterns as follows. The decay in 100mM- K^+ is constant ($K = 0.0084 \text{ min}^{-1}$), representing the maintenance of the active form iE_{II} when K^+ is saturating. (The stability in 400mM- K^+ is identical with that in 100mM). Immediately after removal of K^+ , enzyme decay is rapid ($K = 0.053 \text{ min}^{-1}$), representing the unstable form, iE . However, continued incubation in the absence of K^+ (zero K^+) results in a decrease in the decay rate, finally to $K = 0.0045 \text{ min}^{-1}$. But in zero K^+ the rapidly re-activating form transforms in part to the slowly re-activating form, E (see Fig. 3). Thus it appears that the transition from rapidly re-activating form iE to slowly re-activating form E is also accompanied by a change in stability. If this interpretation is correct, then one prediction is that the rate with which the early fast-decay portion of the zero- K^+ line of Fig. 6 changes to the latter slow-decay portion should equate with the rate of transition of the fast-recovery form iE to the slow recovery form E as calculated from Fig. 3. The decay rate of form iE to form E from Fig. 3 is calculated after subtraction of

that portion due to irreversible decay. This value (0.053 min^{-1}) is obtained from the slope of the zero- K^+ line of Fig. 5. Thus a value of $0.18 - 0.053 = 0.127 \text{ min}^{-1}$ is obtained. This compares well with a rate constant of 0.111 min^{-1} for the rate of change of the slope of the zero- K^+ line of Fig. 6.

Decay rates in zero K^+ at $t = 0$ and $t \rightarrow \infty$ (Figs. 5 and 6) have therefore been assigned to forms iE and E respectively. These are:

Decay of iE , $k = 0.053 \text{ min}^{-1}$

Decay of E , $k = 0.0045 \text{ min}^{-1}$

The constant decay rates at saturating K^+ (Fig. 6) has been assigned to form iE_{II} , $k = 0.0084 \text{ min}^{-1}$. (We have found some variability between different preparations in these rate constants for decay. Thus often the decay in 100mM- K^+ is less than that of the later stages of decay in zero K^+ ; that is, form iE_{II} is more stable than form E .)

Attempts have been made to predict the overall stability at intermediate concentrations of K^+ and intermediate times from calculations of the various proportions of the three conformation states whose stability have so far been covered, and of form E_{II} , whose existence is kinetically feasible but whose stability is difficult to measure directly owing to its transient nature. The results are presented in Table 1, where they are compared with experimentally determined decay rates.

In any enzyme solution the total amount of enzyme possessing conformation state I can be determined by normal mix and assay in saturating K^+ as then all form iE will rapidly re-activate to form iE_{II} and exhibit activity. In order to assay total recoverable enzyme, including E and E_{II} conformations, portions of enzyme must first be subjected to a period of activation that ensures that both the fast and the slow conformation changes have been accomplished before assay.

For the data in Table 1, enzyme samples were incubated in various concentrations of K^+ for 30 min, then assayed in two different ways. A portion was measured immediately in the same K^+ concentration prevailing during the incubation period (this avoids complications of activation during assay). Activities were then corrected for failure to saturate the K^+ requirement for the fast conformation during the assay. The results are presented in Table 1, column 2. Another portion was removed at the same time and incubated in 100mM- K^+ for a further 10 min before assay at 100mM- K^+ . The results are presented in Table 1, column 3. The difference between the two sets of assays is now that the first set of assays has failed to measure any slowly re-activating enzyme forms that have accumulated during the 30 min incubation in subsaturating K^+ , whereas the second set of assays have measured total recoverable enzyme

Fig. 6. Effect of K^+ on the long-term stability of aldehyde dehydrogenase

The enzyme was equilibrated on Sephadex G-25 columns at 4°C with 0.1M-Tris/HCl (pH 8.0)/1mM-mercaptoethanol and KCl at the following concentrations: $\circ$, zero; $\square$, 3; $\bullet$, 7; $\blacktriangle$, 15; $\blacksquare$, 30; ∇ , 100mM. Portions were removed at the times indicated in the Figure and dealt with in a manner identical with that used in Fig. 5.

Table 1. *Distribution between conformations and predicted decay rate after 30 min incubation in various concentrations of KCl*

The enzyme was equilibrated with 100 mM-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol by chromatography on Sephadex G-25 at 4°C. Portions were supplemented with KCl as shown in column (1) and incubated at 25°C for 30 min. Assays were then conducted: for column (2) immediately and at the K⁺ concentration prevailing in the incubation solutions; for column (3), at 100 mM-K⁺ after a 10 min re-activation in 100 mM-K⁺. All other reaction conditions were as described in Fig. 2.

(1) Incubation [K ⁺] (mM)	(2) Activity before 10 min re-activation in 100 mM-K ⁺ *	(3) Activity after 10 min re-activation in 100 mM-K ⁺ †	(4) Fraction possessing conformation I [column (2)/(3)]	(5) Fraction possessing conformation II‡	(6) Predicted decay rate (min ⁻¹)	(7) Experimentally determined decay rate (min ⁻¹)§
1.5	0.135	0.564	0.24	0.18	0.0149	0.0139
3	0.226	0.538	0.42	0.30	0.0197	0.0175
4.5	0.306	0.556	0.55	0.39	0.0255	0.0210
7	0.386	0.502	0.77	0.50	0.0256	0.0239
15	0.471	0.637	0.74	0.68	0.0184	0.0175
30	0.617	0.708	0.87	0.81	0.0157	0.0139
100	0.905	0.923	0.98	0.93	0.0118	0.0088

* Corrected to velocity at 0.1 M-K⁺ during assay.

† Corrected for loss of activity during 10 min re-activation assuming that all enzyme forms possessing state II decay to X with $k = 0.0084 \text{ min}^{-1}$.

‡ Calculated from Fig. 2.

§ Mean values from Fig. 6 and two replicates. F (variance ratio) = 0.44.

activity. The fraction of enzyme in the rapidly re-activating form (possessing state I) after 30 min incubation in various K⁺ concentrations is presented in Table 1, column 4. Assuming as the model does that conformation change I is independent of change II, then the fraction of enzyme possessing or not possessing state I calculated above can be distributed between those possessing and not possessing state II by using the data of Fig. 2. This has been done in Table 1, column 5. Table 1 then compares the decay rate predicted from the calculated populations of the four conformations assuming that all forms having conformation state II are equally unstable, with the actual decay rates obtained from Fig. 6 at 30 min. As can be seen, the rates are in general agreement. In particular, the order of stabilities follows the same pattern, with stability highest in zero or 100 mM-K⁺ and lowest at 7 mM-K⁺.

It may be imagined that 30 min after a change in cation status, both conformation squares would be nearing equilibrium, in which case the data of Table 1, column 4, presenting as it does the fraction of enzyme in the fast-re-activating form and therefore possessing state I, would enable a calculation of K_s^I for K⁺ for state I, the slow conformation change. However, this cannot be so simply achieved. This is because form I_2E decays to the irreversibly inactivated state at a rate comparable with the rate constant for the transformation of form E into form I_2E . Thus, whenever the decay from form I_2E to form X is significant, then true equilibrium between all potentially active species is not reached, and the

fraction of molecules left possessing state I will be lower than indicated by the equilibrium round square I. In spite of these considerations, the fraction of enzyme possessing state I is somewhat greater than that possessing state II at all incubation concentrations of K⁺. This then indicates that the K⁺ requirement for inducing conformation state I is less than that for state II.

Although the kinetic and stability data reported above are on impure enzyme preparations, the basic features of activity and stability in relation to cation status are essentially similar in the homogeneous enzyme preparations used for the spectroscopic studies reported below. The behaviour of homogeneous enzyme is complicated by its marked instability even in saturating K⁺ unless high concentrations of polyhydric alcohol are also present (Bradbury & Jakoby, 1972). With homogeneous enzyme, rapid changes in activity in response to changes in cation concentration are again observed as evidence of a fast conformation change. In the presence of ethanediol, in the absence of K⁺, there is once again a biphasic decay curve. Prolonged incubation in the absence of K⁺ and polyhydric alcohol results in an enzyme form whose activity slowly recovers on returning to K⁺. That is, there is once again evidence of an additional slow conformation change.

A difference does occur between homogeneous enzyme (and an occasional crude preparation) and the more usual crude preparations reported above. This is that, for homogeneous enzyme and an occasional

Fig. 7. *U.v. difference spectra of aldehyde dehydrogenase in activating and non-activating environments*

The enzyme was equilibrated on Sephadex G-25 columns against 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol/10% (v/v) ethanediol. Curves (i) are baselines; curves (ii) are scans immediately after adding perturbants; curves (iii) show recovery of baseline by equalizing perturbants in reference and sample cells at 0.1 M. Perturbants are: for (a), sample cell to 0.1 M-KCl; for (b), sample cell to 0.1 M-LiCl; for (c), sample cell to 0.1 M-KCl and reference cell to 0.1 M-LiCl. The protein concentration was initially approx. $2.3 \mu\text{M}$, giving an A_{280} of 1.4.

crude preparation, incubation with NAD^+ can eradicate the acceleration of reaction velocity observed when enzyme has been preincubated in the absence of K^+ . The dual nature of the activation process in these cases is clearly seen because, although NAD^+ can induce the slow-re-activation process, it cannot induce the fast-re-activation process, which still requires K^+ .

Cation-induced spectral changes

Direct evidence of K^+ -induced conformation changes have been sought by investigation of u.v.-absorption spectra of aldehyde dehydrogenase in various cation environments.

Fig. 7 shows a series of u.v. difference spectra produced with homogeneous aldehyde dehydrogenase

in different univalent-cation environments, in a background buffer consisting of 0.1 M-Tris/HCl (pH 8.0)/10% (v/v) ethanediol/1 mM-mercaptoethanol, in which the enzyme is not active. The results shown in Fig. 7(a) are produced when concentrations of K^+ optimal for activity are used to perturb enzyme. The addition of K^+ has a dramatic effect on the absorbance difference spectrum with a negative peak at 295 nm ($\Delta\epsilon_{295}/\epsilon_{279} = 0.011$), and two positive peaks at 279 nm ($\Delta\epsilon_{279}/\epsilon_{279} = 0.012$) and 288 nm ($\Delta\epsilon_{288}/\epsilon_{279} = 0.0087$). Fig. 7(b) shows a similar experiment except that non-activating Li^+ is used as perturbant. The addition of Tris^+ produces only a small difference spectrum, with a maximum at 287 nm ($\Delta\epsilon_{287}/\epsilon_{279} = 0.004$). Fig. 7(c) is a difference spectrum generated by

Fig. 8. U.v. difference spectra of aldehyde dehydrogenase with Tl^+ as perturbant

The enzyme was equilibrated on Sephadex G-25 columns against 0.1 M-Tris/ HNO_3 (pH 8.0)/1 mM-mercaptoethanol/10% (v/v) ethanediol. Curve (a), sample cell titrated to 5 mM- TlNO_3 ; curve (b), sample cell and reference cell titrated to 4 mM- TlNO_3 and 4 mM- KNO_3 respectively; curve (c), sample cell and reference cell titrated to 40 mM- TlNO_3 and 40 mM- KNO_3 respectively. The protein concentration initially approx. 2.3 μM . Difference spectra were corrected for difference in absorbance of nitrate and for a flat baseline.

aldehyde dehydrogenase in buffer plus non-activating Li^+ versus buffer plus activating K^+ . An essentially similar difference spectrum is seen to Fig. 7(a). The difference spectrum of Fig. 7(c) can be understood as a combination of the two difference spectra of 7(a) and 7(b). As Li^+ is now in the reference cell the $\Delta\epsilon$ at 287 nm of Fig. 7(b) now causes a shift of the 288 nm positive maximum of Fig. 7(a) to a longer wavelength.

Fig. 8 shows u.v. difference spectra of aldehyde dehydrogenase generated with Tl^+ . Experiments were performed against a background buffer consisting of 0.1 M-Tris/ HNO_3 (pH 8.0)/10% (v/v) ethanediol/1 mM-mercaptoethanol. Because of the absorbance of the NO_3^- anion, corrections have been made for its contribution to the difference spectrum where Tl^+ is added as its nitrate salt. Fig. 8, curve (a), is a difference spectrum obtained when enzyme is perturbed with Tl^+ at a concentration optimal for activity. The similarity to curves obtained with K^+ as the perturbant is obvious ($\Delta\epsilon_{295}/\epsilon_{279} = 0.0051$, $\Delta\epsilon_{288}/\epsilon_{279} = 0.0074$). Fig. 8, curve (b), is a difference spectrum when enzyme in 4 mM- Tl^+ in the sample cell is compared with enzyme in equimolar K^+ in the reference cell, both in an identical background buffer. Here a difference spectrum can still be seen, though much suppressed. This reflects the higher affinity of enzyme for Tl^+ (Bostian *et al.*, 1975); that is, there is more

Fig. 9. U.v. difference spectra of aldehyde dehydrogenase at various K^+ concentrations

The Figure shows immediate scans of enzyme when the sample cell was perturbed to; (a), zero; (b), 3; (c), 6; (d), 12; (e), 27; (f), 57; (g), 102 mM-KCl. The insert shows a double-reciprocal plot of variation of $(\Delta\epsilon_{288} - \Delta\epsilon_{295})$ with KCl concentration. All other conditions were as described for in Fig. 7.

active enzyme in the Tl^+ cell. Fig. 8, curve (c), shows that, at concentrations of univalent cation closer to saturation, the characteristic active-versus-non-active difference spectrum appear to be further suppressed. The new features of this difference spectrum may reflect another non-active state induced at high Tl^+ concentrations that are inhibitory when compared with the concentration of Tl^+ optimal for activity (Bostian *et al.*, 1975).

Figs. 7 and 8 demonstrate that a characteristic difference spectrum is produced whenever enzyme in an activating environment (K^+ or Tl^+) is compared with enzyme in a non-activating environment (Li^+ or Tris^+).

The concentration-dependence of the K^+ -induced u.v. difference spectrum is demonstrated in Fig. 9 by using $\Delta\epsilon_{288} - \Delta\epsilon_{295}$ as a measure of difference absorbance. A plot of this spectral parameter against $[\text{KCl}]$ is hyperbolic, with a value of 6.2 mM- K^+ providing 50% of the maximum spectral change. The mean of three determinations was 6.0 mM and is not significantly different at the 10% level ($P = 0.1$) from the K_s for K^+ -inducing activity.

The rate of change in absorbance of enzyme on the re-addition of KCl to enzyme just previously depleted of K^+ (Fig. 7a) was monitored by measuring A_{279} against a bovine serum albumin reference. The large

change on supplementation to saturating KCl occurs within 10s, the time necessary to mix the sample and return to the spectrophotometer. Perturbation of this sample with LiCl (to 0.1M) causes an almost total reversal of this K^+ -induced absorbance change, again within the time required for mixing. Similar additions of LiCl to an enzyme in saturation K^+ results in a rapid inhibition of enzyme activity (Fig. 1). The rate of absorbance change caused by KCl, or reversed by LiCl, occurs rapidly enough to correlate with the time required for adjustment of enzyme activity on identical changes in cation environment, as determined by stopped-flow analysis. Unfortunately more critical evaluation of the rate of appearance of the difference spectrum by rapid mixing and stopped flow is not possible, owing to the low magnitude of the absorbance change.

Fig. 10(a) shows that, when a difference spectrum such as produced in a Fig. 7(a) is scanned repeatedly over 20min, small increases are observed in the difference spectrum of the initial scan. These changes cannot be attributed to the tail-end of a supposed first-order process producing the initial difference spectrum. Rather, if the rapid change is first-order, then these slow changes must represent an additional process having a considerably smaller rate constant.

Because neither the activity nor the absorbance of the enzyme in K^+ measured against a static reference changes over these time periods, this implies a slow change in the reference enzyme of Fig. 10(a). Fig. 10(b) shows that, after a prolonged incubation in Tris/HCl, the addition of KCl to the reference enzyme only slowly and incompletely suppresses the difference spectrum. In essence the studies show that,

Fig. 10. Increase in K^+ -induced u.v. difference spectra of aldehyde dehydrogenase with time (a): (i) is the baseline scan of enzyme in buffer in reference and sample cells [and in (b) also]. Sample cells were then titrated to 0.1 M KCl and the spectrum scanned at the following times: (ii) zero; (iii) 2.3; (iv) 4.8; (v) 7 min. (b) The reference sample from (v) was then also titrated to 0.1 M-KCl, and the spectrum scanned at the following times: (vi) zero; (vii) 2.5; (viii) 4; (ix) 10 min. All other conditions were as described for Fig. 7.

on removing activating ions, an enzyme conformation is established that can be altered within 10 s by the re-introduction of activator (Figs. 7 and 8). If, however, the enzyme is allowed to incubate for several minutes in a non-activating environment (Fig. 10), then several minutes are required for a conformation change to occur on returning the enzyme to an activating environment. This behaviour exactly parallels the loss and gain of activity after the same changes in univalent-cation environment and give substance to the proposed slow conformation transition.

Although it has been proposed that the slowly recovering species, E, is accumulating in the reference cell of Fig. 10(a) at the expense of ${}_1E$, we prefer to consider forms ${}_1E$ and E as having identical spectra and to attribute the enhanced spectral shifts on repeated scanning to the accumulation of irreversibly inactivated enzyme in the reference cell. Our preference for this explanation is based on the following consideration. Even after prolonged incubation of both reference and sample enzyme in K^+ , the baseline is not completely restored. The residual difference is equal in magnitude to the difference between the first and last perturbed scans of Fig. 10(a). This, then, is more consistent with the accumulation in the reference cell of an irreversibly changed (inactivated) form than a form that can be slowly re-activated to the form that exists in the sample cell. This is more clearly seen if an enzyme sample is allowed to decay in a Tris/HCl buffer until essentially no activity can be recovered on returning to K^+ . When this enzyme is again freed from K^+ , in order to repeat the experiment of Fig. 10, there is virtually no increase in the $\pm K^+$ difference spectra after the first scan after perturbation of sample enzyme with K^+ , and eventually an almost complete restoration of baseline on perturbation of reference enzyme with K^+ . In this case we argue that most enzyme is irreversibly inactivated before the spectral measurements begin, and therefore no more can be inactivated in the Tris environment of the reference cell than in the Tris plus K^+ environment of the sample cell.

With reference to the last point, it would appear that decay to the irreversibly inactive form does not destroy the ability of K^+ to induce conformation transitions, rather that such transitions cannot result in gain or loss of activity because of some overriding denaturing change.

In u.v.-difference experiments where polyhydric alcohol was not included in the buffer system, variations from the previous spectra were observed. These are shown in Figs. 11(a) and 11(b). Firstly the enzyme $\pm KCl$ perturbation spectra retains its general appearance, and the magnitude of $\Delta\epsilon/\epsilon_{279}$ values were approximately the same. However, there is a greater increase in absorbance difference on repeated scanning (Fig. 11b). This is to be expected, since, in

Fig. 11. U.v. difference spectra of aldehyde dehydrogenase in the absence of polyhydric alcohol

The enzyme was equilibrated on Sephadex G-25 columns against 0.1 M-Tris/HCl (pH 8.0)/1 mM-mercaptoethanol. (a) Curve (i), sample cell titrated to 0.1 M-KCl; (ii) sample cell titrated to 0.1 M-LiCl; (iii) sample and reference cell titrated to 0.1 M-LiCl and 0.1 M-KCl respectively. The spectrum was scanned immediately after adding perturbation. (b) The enzyme was as in (a), in which the sample cell was titrated to 0.1 M-KCl and the spectrum scanned at the following times: (i), zero; (ii), 2.5; (iii), 6; (iv), 9.5 min. The protein concentration was approx. $2.65 \mu\text{M}$, giving an A_{280} of 1.6. The difference spectra were corrected for the flat baseline.

the absence of polyhydric alcohol, decay of enzyme to irreversibly inactive enzyme, which has a slightly different u.v. spectrum (see above) is considerably more rapid (Bradbury & Jakoby, 1972). A much greater positive difference occurs at wavelengths in the region below 265 nm. In addition, in all cases where there is an imbalance of univalent-cation salt concentration between sample and reference, a very large positive difference occurs in the region below 265 nm. This is probably due to a strong electrostatic

effect on exposed chromophore. For the enzyme $\pm$ LiCl difference spectrum, although the magnitude of the 287 nm difference maxima remains relatively small compared with KCl maxima, it displays this large positive difference beginning just below 280 nm. For KCl/enzyme-against-LiCl/enzyme difference spectra, in which univalent-cation salt concentration is balanced, this results in a large broad positive peak from 290 to 255 nm containing the fine details of the previous two difference spectra.

Attempts to simulate the active-versus-non-active difference spectra of aldehyde dehydrogenase with perturbations of model compounds are shown in Fig. 12. Curve (a) is a charge-induced difference spectrum of indol-3-ylethylamine (Andrews & Forster, 1972) and curve (b) is a solvent-perturbation red shift of *N*-acetyl-L-tyrosine ethyl ester (Herskovits & Sorensen, 1968). Curve (c) is the sum of curves (a) and (b). The difference spectra for enzyme in Tris versus enzyme in Tris plus K^+ can be seen to be consistent with conformational changes resulting in aromatic amino acid residues being placed in a different environment. The negative difference at 295 nm is indicative of a charge-perturbation blue shift of the indole group of tryptophan similar to curve (a) of Fig. 12. The positive difference maxima at 288 nm and 279 nm indicate a polarity-perturbation red shift of tyrosine (Fig. 12, curve b). A combination of these two separate effects shown in curve (c) of Fig. 12 gives a spectrum very similar to that observed experimentally for aldehyde dehydrogenase in activator versus non-activator environments. The ability of K^+ to induce a relatively large red shift in tyrosine absorbance indicates a 'burial' of this chromophore in a more hydrophobic environment, presumably associated with an enzyme conformation change. This effect occurs whenever the active enzyme in either K^+ or Tl^+ is compared with an inactive enzyme in $Tris^+$ or Li^+ .

The ability of activators Tl^+ or K^+ to make more positive the net charge with respect to at least one residue of tryptophan may reflect a binding of activator cations in close proximity to the indole moiety. The fact that Li^+ fails to induce this spectral change would have to be interpreted as arising from a difference in binding sites. But Li^+ is a competitive inhibitor of K^+ in enzyme activity and is a similar ligand, and this would seem to indicate an ability to bind at the same sites. An alternative explanation of a charge-induced shift, and one consistent with our interpretations of the protein-conformational basis of enzyme activation by K^+ , is that K^+ and Li^+ bind at the same site, which is, however, distant from the critical tryptophan residue, but only K^+ is able to induce the conformation change in which appropriately orientated negatively charged group(s) are displaced from, or positively charged group(s) are repositioned with respect to, the tryptophan moiety.

The present studies have shown that activity, stability and u.v.-spectral properties of aldehyde dehydrogenase are all affected by cation status. K^+ -induced changes in activity and stability were not affected by changes of approx. 10-fold in protein concentration. Spectral changes induced by K^+ were independent of protein concentration over an approx. 5-fold change in concentration. Thus there is no evidence of association/dissociation of enzyme subunits in these phenomena. The response of the enzyme to changes in cation status with respect to all three characteristics depends critically on the length of time that it has been kept in a non-activating environment. Rapid transfers of enzyme between activating and non-activating environments result in rapid changes in all three characteristics. However, if the enzyme is held in a non-activating environment, slow changes occur, after which the enzyme responds much more slowly to the reintroduction of activating ions. There is no significant difference between the K^+ requirement for inducing the rapid changes in activity, stability and u.v. absorption, with mean values of 7.5 mM, 5.5 mM and 6.0 mM respectively for the concentration of K^+ required for inducing 50% of the stated phenomena.

In addition, correlation has been obtained for the nature of the ions that activate and also provide a characteristic u.v.-absorption spectrum; thus K^+ and

Fig. 12. U.v. difference spectra of model compounds (a) Scan of indol-3-ylethylamine/HCl in sample cell and indol-3-ylethylamine in reference cell; the solvent was water (from Andrews & Forster, 1972). (b) *N*-Acetyl-L-tyrosine ethyl ester in 0.1 M-KCl/0.01 M-potassium phosphate, pH 6.8, with sample cell perturbed with 20% (w/v) sucrose (from Herskovits & Sorensen, 1968). (c) Addition of (a) and (b).

Fig. 13. General model for K^+ -activation involving two K^+ -induced conformation transitions

Conformation transitions I and II represent slow and fast conformation transitions respectively. ${}_1E$, inactive rapidly re-activating conformer; E and E_{II} , inactive slowly re-activating conformers. Only conformers possessing both transitions I and II (${}_1E_{II}$) are active; X , irreversibly inactivated conformer.

Tl^+ both activate and induce a similar absorption spectrum, which differs from that produced by the non-activating ions $Tris^+$ and Li^+ .

We have so far found no explanation for these phenomena consistent with all the data other than to propose that they are the result of two separate cation-induced conformation changes.

The following argument points out some consequences of a scheme of two conformation changes both induced by K^+ .

Both K^+ -induced and K^+ -stabilized conformation changes are considered. If K^+ binding at a single site is responsible for both conformation changes, and if these changes occur independently, then a general model for the interconversion of four active or potentially active conformations is as given in Fig. 13.

With a ligand-induced or stabilized conformation change the fractional saturation of binding sites, $\bar{Y}$, by using constants designated in Fig. 13 for transitions involving the loss or gain of conformation state II is:

$$\bar{Y} = \frac{K^+}{K^+ + \frac{1 + K_6}{\frac{1}{K_7} + \frac{K_6}{K_4}}}$$

This is a hyperbolic form of the equation given by Frieden (1970) and is also equivalent to that of Monod *et al* (1965) when there is no interaction between binding sites ($n = 1$).

Activity in excess of $V_{max.}/100$ could be detected, but activity in the absence of K^+ is zero. Thus $K_6 > 100$ and the constant term in the denominator approximates to:

$$\frac{K_6}{\frac{1}{K_7} + \frac{K_6}{K_4}}$$

It is unlikely that a substantial proportion of enzyme remains in the inactive form in the presence of saturating concentrations of activator, so $K_5 < 0.1$. Thermodynamic requirements demand that:

$$K_6 K_7 = K_4 K_5$$

But as $K_6 > 100$ and $K_5 < 0.1$, then $K_4 > 1000 K_7$. Thus:

$$\frac{K_6}{K_4} < 0.1 \frac{1}{K_7}$$

and the constant denominator term approximates to $K_6 K_7$ or $K_4 K_5$. As the apparent dissociation constant (K_s) for K^+ in the fast square involving alterations in conformation state II = 7.5 mM and $K_5 < 0.1$, then $K_4 > 75$ mM.

But the equilibrium described by K_4 is also included in the thermodynamic square in which changes of conformation state I are involved. Now the same arguments can be made for K_2 and K_3 as were made for K_5 and K_6 . But the estimation of K_4 at 75 mM places a constraint on the overall K_s for square I, and if $K_3 = K_6 > 100$, then the K_s for square I = $100 \times 0.075 = 7.5$ M.

In this case, form E would appear to bind K^+ insignificantly at concentrations optimal for activity. The pathway for re-activation of form E would then essentially be via K^+ -free forms ${}_1E$ or E_{II} and ${}_1E_{II}$, with these forms stabilized by the relatively tight binding round square II. Alternatively, if enzyme forms that have lost conformation state II can still bind K^+ significantly at physiological concentration, then this may require the removal of the assumption that a single K^+ -binding site is responsible for both conformation transitions.

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