The binding of NADH to cytoplasmic aldehyde dehydrogenase after modification with *p*-nitrophenyl dimethylcarbamate

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Cytoplasmic aldehyde dehydrogenase may be modified by reaction with *p*-nitrophenyl dimethylcarbamate to give a stable E–X–CO–NMe₂ species that is an analogue of the usual labile acyl-enzyme involved in the enzyme's reactions. [X is derived from an enzymic nucleophilic group.] This species still contains the tightly bound NADH that is present in the native enzyme. When further NADH binds to E–X–CO–NMe₂ its fluorescence is enhanced over 4 times more than when it binds to unmodified enzyme; this fluorescence is completely unaffected by high propionaldehyde concentration and only slightly affected by *p*-nitrobenzaldehyde. The modified species has 1.0 NADH binding site in the absence of Mg²⁺ and 1.67 sites in its presence. The rate of dissociation of E–X–CO–NMe₂·NADH is biphasic (*k* 3.4 and 1.8 min^{−1}) and is considerably lower than that of E·NADH; the presence of Mg²⁺ slows the process even more (*k* 0.47 and 0.37 min^{−1}). The implications of these studies towards a greater understanding of the nature of aldehyde dehydrogenase-catalysed reactions are discussed.

INTRODUCTION

In the preceding paper (Kitson, 1989*b*) it was reported that sheep liver cytoplasmic aldehyde dehydrogenase reacts slowly with *p*-nitrophenyl (PNP) dimethylcarbamate to give *p*-nitrophenoxide and a modified form of the enzyme, E–X–CO–NMe₂ (where X, possibly sulphur, derives from the enzymic group that acts nucleophilically towards the modifier). The modified enzyme can be readily isolated from product and excess modifier by gel filtration as the rate of hydrolysis of E–X–CO–NMe₂ is virtually zero. In the course of the enzyme's normal catalytic reaction it is thought that a hydride ion is transferred from enzyme-bound aldehyde (perhaps as a thiohemiacetal) to NAD⁺, thereby producing NADH and an acyl-enzyme, E–X–CO–R. Usually the latter is hydrolysed very rapidly and, after dissociation of NADH, the enzyme is then free to carry out another catalytic cycle. Only in a few special cases, such as when R–CO– is *p*-dimethylaminocinnamoyl, is the acyl-enzyme physically observable, and even then it is not stable for long against hydrolysis (Dunn & Buckley, 1985). It was theorized that the species described above derived from aldehyde dehydrogenase and PNP dimethylcarbamate ideally might differ from the enzyme's typical acyl-enzyme intermediate only in having a much longer lifetime. Study of the physical properties of E–X–CO–NMe₂ (such as, for example, how it interacts with NADH, NAD⁺, Mg²⁺ etc.) might therefore illuminate the character of the usual acyl-enzyme, for which such studies are complicated or precluded by its short lifetime. This was the aim of the present work, as is described below.

EXPERIMENTAL

Materials

The preparation and assay of sheep liver cytoplasmic aldehyde dehydrogenase, the synthesis of PNP

dimethylcarbamate and the source of other chemicals were all as indicated in the preceding papers (Kitson, 1989*a,b*).

Methods

Enzyme assays and the isolation of E–X–CO–NMe₂ were carried out as indicated in the preceding paper (Kitson, 1989*b*).

Fluorescence spectrum of enzyme-bound NADH. This was determined at approx. 20 °C with a Farrand mark I spectrofluorimeter equipped with a micro-cell attachment. Excitation was at 350 nm and 10 nm slits were used. Fluorescence readings were taken at 5 nm intervals from 380 to 510 nm. The spectrum of added NADH was determined in 1 ml samples of 50 mM-Tris/HCl buffer, pH 7.4, with an E–X–CO–NMe₂ concentration of 2.88–2.92 μM; [NADH] was 10 μM, [Mg²⁺] 0 or 10 mM, [propionaldehyde] 0 or 100 mM, and [*p*-nitrobenzaldehyde] 0, 20, 50 or 100 μM. The last-mentioned compound was added in 10 μl of ethanol; the same volume of ethanol was added to the control spectrum determination.

NADH fluorescence titrations. The binding of NADH to aldehyde dehydrogenase was monitored fluorimetrically as described by Hart & Dickinson (1977). Excitation was at 366 nm and a narrow-band-pass interference filter, λ_{max}. 430 nm, was placed before the detector. Sequential additions of NADH (in negligibly small volume, 5 or 10 μl) were made to 4 ml of 50 mM-Tris/HCl buffer, pH 7.4, or 50 mM-sodium phosphate buffer, pH 7.4, at 25 °C, and also to identical solutions containing native enzyme or E–X–CO–NMe₂ and in some cases (with Tris buffer) Mg²⁺ (2.5, 10 or 20 mM).

NADH-displacement studies. These were performed with a recording filter fluorimeter of the type described by Dalziel (1962). A solution of E–X–CO–NMe₂

Abbreviations used: PNP, *p*-nitrophenyl; E–X–CO–NMe₂, enzyme after modification by PNP dimethylcarbamate.

(1.27 μM) in 4 ml of 50 mM-sodium phosphate, pH 7.4, or 200 mM-Tris/HCl buffer, pH 7.4, containing 10 mM- Mg^{2+} , was mixed at 25 °C with 10 μl of aqueous NADH (to give 3 μM) and the fluorescence was recorded until no more increase was evident. At this point 25 μl of aqueous NAD^+ (to give 1 mM in the final mixture) was added rapidly on a glass nail and the subsequent fluorescence changes were recorded, starting within a few seconds and continuing until there was no further change.

RESULTS AND DISCUSSION

Presence of tightly bound nucleotide in aldehyde dehydrogenase

Hart & Dickinson (1983) reported that samples of sheep liver cytoplasmic aldehyde dehydrogenase, prepared by the method of Dickinson *et al.* (1981) (as also used in the present work), contain various amounts of tightly bound NADH. They discuss whether or not this material is actually associated with aldehyde dehydrogenase itself or with some minor impurity that happens to travel with the enzyme through its lengthy purification procedure. In the present work it was found that the fluorescence of the modified enzyme (E-X-CO-NMe_2) is about half as intense again as that of the native enzyme (determined under identical conditions) and shows a small but reproducible shift in λ_{max} from 425 to 420 nm. The result shows clearly that the tightly bound NADH is indeed associated with aldehyde dehydrogenase. It would be most unlikely for a minor and specific chemical modification of this enzyme to have such a significant effect on the fluorescence of NADH bound to some other contaminating protein. Why tightly bound NADH should be present in aldehyde dehydrogenase, why it should vary so much from one preparation to another, and how it avoids dissociation from the enzyme during gel filtration or prolonged dialysis remain matters for speculation (see Hart & Dickinson, 1983).

Fluorescence spectrum of NADH when bound to E-X-CO-NMe_2

Fig. 1 shows the fluorescence spectrum obtained when NADH is added to E-X-CO-NMe_2 . (The fluorescence due to the tightly bound NADH already present is small on the scale used for Fig. 1.) When NADH binds to the modified enzyme its fluorescence emission maximum is shifted from 460 to 420 nm and is greatly enhanced in magnitude. (Note the 10-fold difference in scales for fluorescence of free and bound NADH in Fig. 1.) The enhancement factor is much more than that observed with native enzyme in a similar experiment (Dickinson, 1985). The apparent λ_{max} of approx. 440 nm observed by Dickinson (1985) arises by overlap of the fluorescences of bound NADH and free NADH. In the present work, however, the fluorescence of free NADH is negligible compared with the greatly enhanced fluorescence of bound NADH and thus the λ_{max} of 420 nm for the latter is genuine.

The dramatic effect that modification by PNP dimethylcarbamate has on the fluorescence of enzyme-bound NADH suggests that the site of modification may be very close to the NADH-binding site, which in turn is consistent with the notion that PNP dimethylcarbamate reacts with the enzymic nucleophile involved in the dehydrogenase activity of the enzyme. With native

Fig. 1. Fluorescence-emission spectra of NADH in the presence and in the absence of E-X-CO-NMe_2 , propionaldehyde and Mg^{2+}

Solutions were made up in 50 mM-Tris/HCl buffer, pH 7.4, with 10 μM -NADH and 2.88 μM - E-X-CO-NMe_2 as follows: Δ , NADH control (no enzyme); $\circ$, NADH and enzyme, or NADH, enzyme and 100 mM-propionaldehyde (i.e. the latter had no effect on the spectrum); $\square$, NADH, enzyme and 10 mM- Mg^{2+} ; $\blacksquare$, NADH, enzyme, 10 mM- Mg^{2+} and 100 mM-propionaldehyde. Note that the relatively low level of fluorescence of NADH alone was multiplied by 10 before plotting (Δ).

enzyme, Dickinson (1985) found that the fluorescence spectrum of enzyme-bound NADH was altered by high concentrations of propionaldehyde and used this as evidence for the formation of a ternary $\text{E} \cdot \text{NADH} \cdot \text{aldehyde}$ complex. Presumably the aldehyde binds in this complex by interacting covalently and reversibly with the enzymic nucleophile. However, if this enzymic group is that which is modified in E-X-CO-NMe_2 , then it could no longer interact with aldehydes in this way and it would not be surprising if there were no change in the fluorescence of $\text{E-X-CO-NMe}_2 \cdot \text{NADH}$ when aldehyde is added. This is indeed found to be the case; the spectrum of $\text{E-X-CO-NMe}_2 \cdot \text{NADH}$ in Fig. 1 is exactly the same in the presence of 100 mM-propionaldehyde as in its absence.

Fig. 1 also shows that the already strong fluorescence of $\text{E-X-CO-NMe}_2 \cdot \text{NADH}$ is enhanced even more in the presence of Mg^{2+} . Under these circumstances the addition of propionaldehyde results in a small but reproducible decrease in the intensity of fluorescence (a point returned to below).

Dickinson (1985) found that the fluorescence emission of NADH bound to native enzyme was markedly quenched when low concentrations (5–50 μM) of *p*-nitrobenzaldehyde were added. With E-X-CO-NMe_2 , however, Fig. 2 shows that *p*-nitrobenzaldehyde (20–100 μM) has only a small quenching effect on the fluorescence of bound NADH. The very tight binding of *p*-nitrobenzaldehyde to $\text{E} \cdot \text{NADH}$ observed by

Fig. 2. Fluorescence-emission spectra of NADH in the presence of E-X-CO-NMe₂ and *p*-nitrobenzaldehyde

Solutions were made up with 10 μ M-NADH and 2.92 μ M-E-X-CO-NMe₂ in 50 mM-Tris/HCl buffer, pH 7.4, containing various concentrations of *p*-nitrobenzaldehyde, which were (from top spectrum to bottom) 0 μ M, 0 μ M (duplicate), 20 μ M, 50 μ M and 100 μ M. A solution of 10 μ M-NADH gave a fluorescence of 0.04 unit at 460 nm.

Fig. 3. Fluorescence titration of native and modified aldehyde dehydrogenase with NADH

Small volumes of NADH solution were added incrementally to 50 mM-sodium phosphate buffer, pH 7.4, in the presence and in the absence of enzyme, and the fluorescence was determined as described in the Experimental section. □, No enzyme; ○, native enzyme (1.0 μ M); ●, E-X-CO-NMe₂ (1.0 μ M).

Dickinson (1985) might be due to reversible covalent interaction with the critical group X, whereas with E-X-CO-NMe₂·NADH only a relatively weak hydrophobic interaction between the aromatic aldehyde and the enzyme's aldehyde-binding site would be possible. Thus these observations too are not inconsistent with the notion that modification by PNP dimethylcarbamate involves the enzyme's essential group.

NADH titrations with native enzyme and E-X-CO-NMe₂

Fig. 3 shows the results of fluorescence titrations (carried out as indicated in the Experimental section) during which NADH is added incrementally to samples of native enzyme and to E-X-CO-NMe₂. The Figure makes very clear that the modified form of the enzyme enhances the fluorescence of NADH much more than does the native form. It can be calculated that when each molecule of NADH binds to E-X-CO-NMe₂ its fluorescence is enhanced 46-fold. For E-X-CO-NMe₂ the data were re-expressed as a plot of $1/(1-R)$ against $[NADH]/R$ where R is the fractional saturation of the binding sites with NADH; this is a transformation of the Scatchard relationship as derived by Gutfreund (1972). A good straight line was obtained, the number of NADH-binding sites was deduced to be 0.9 per tetrameric enzyme molecule and the dissociation constant for E-X-CO-NMe₂·NADH was found to be 0.43 μ M. Averages of these and several more determinations were: number of sites 1.0; K 0.36 μ M. Thus the modified enzyme binds NADH rather more tightly than does native enzyme, for which the dissociation constant is reported to be 1.1 μ M (Bennett *et al.*, 1983).

The strong fluorescence of E-X-CO-NMe₂·NADH reported here presents something of a problem in view of

the thesis advanced by Dickinson and colleagues in a series of papers that the E-acyl·NADH complex produced during the action of the enzyme as a dehydrogenase is non-fluorescent (Hart & Dickinson, 1982; Dickinson, 1985, 1986; Dickinson & Haywood, 1986). This is proposed because there is a rapid burst of A_{340} but a significantly slower increase in fluorescence; the latter process is thought to be consequent on the hydrolysis of E-acyl·NADH to give E·NADH. An explanation for the different fluorescence properties of E-X-CO-NMe₂·NADH and the usual E-acyl·NADH species might be found in the involvement of different enzyme conformations. During the operation of the enzyme's usual catalytic cycle, at least two (possibly three) different enzyme conformations are thought to occur (Bennett *et al.*, 1982; Motion *et al.*, 1984; Blackwell *et al.*, 1987). In particular, the binding of NAD⁺ and aldehyde induces a certain specific conformation in the enzyme that facilitates the hydride transfer between the two substrates. After hydrolysis of the acyl-enzyme the enzyme conformation relaxes to its original state to allow the dissociation of NADH. However, the E-X-CO-NMe₂ used here is necessarily prepared in the absence of NAD⁺ (since reaction of PNP dimethylcarbamate with the enzyme is drastically slowed by NAD⁺; Kitson, 1989b) and thus the enzyme conformation in this species cannot be the same as that described above, which is encountered in the dehydrogenase catalytic cycle. It seems therefore that the specifically non-fluorescent conformer of E-acyl·NADH is only attainable in the forward direction of the normal dehydrogenase reaction, and this idea fits in well with the detailed mechanistic pathway proposed by Bennett *et al.* (1982). An alternative explanation for the difference in fluorescence between E-X-CO-R·NADH and E-X-

CO-NMe₂·NADH is suggested as follows. With the normal acyl-enzyme there may be a strong interaction between the polarized carbonyl group of the acyl moiety and the hydrogen of NADH that has just been transferred in the redox step of the reaction:

This might account for the quenching of the NADH fluorescence. However, in E-X-CO-NMe₂ the partial positive charge on the carbonyl carbon atom is delocalized by resonance such that it largely resides on the nitrogen atom instead:

Consequently there may no longer be the same mechanism for quenching of the NADH fluorescence. A potent effect of the nitrogen atom in this way is not implausible; the same factor certainly has an enormous effect on the interaction of E-X-CO-NMe₂ with water compared with a normal acyl-enzyme.

Effect of Mg²⁺ on NADH fluorescence titrations

The effect of 20 mM-Mg²⁺ on the titration of E-X-CO-NMe₂ with NADH is shown in Fig. 4(a). As mentioned in connection with Fig. 1, Mg²⁺ augments the already strong fluorescence of bound NADH; it also renders the early part of the titration profile sufficiently linear for an estimate of the number of NADH-binding sites to be made by a simple extrapolation procedure of the linear sections of the profile as shown in the Figure. In the absence of Mg²⁺ the titration profile is too curved for this procedure to be applied; instead the number of binding sites is found from the plot of 1/(1-R) versus [NADH]/R (Gutfreund, 1972) as shown in Fig. 4(b). The average for six such determinations was a value of 1.0 binding site in the absence of Mg²⁺ and 1.67 binding sites in the presence of Mg²⁺. (Essentially the same titration profiles were obtained with 2.5 mM, 10 mM- and 20 mM-Mg²⁺.) Likewise the average values of the dissociation constant of NADH were found to be 0.36 μ M and 0.087 μ M in the absence and in the presence of Mg²⁺ respectively.

Previous studies with native enzyme have similarly found that Mg²⁺ tightens the binding of NADH considerably, but they have also shown that Mg²⁺ does not increase the number of NADH-binding sites (Bennett *et al.* 1983; Dickinson & Haywood, 1986), in contrast with the unequivocal result reported here that, with E-X-CO-NMe₂, the number of sites is increased almost 2-fold. This difference prompted a re-investigation of the native-enzyme case during the present work; the results

Fig. 4. Fluorescence titration of E-X-CO-NMe₂ in the presence and in the absence of Mg²⁺

(a) Small volumes of NADH solution were added incrementally to 50 mM-Tris/HCl buffer, pH 7.4, in the presence and in the absence of enzyme, and the fluorescence was determined as described in the Experimental section. □, No enzyme; ○, E-X-CO-NMe₂ (0.84 μ M); ●, E-X-CO-NMe₂ (0.84 μ M) and Mg²⁺ (20 mM). (b) The data in (a) are re-expressed as a plot of 1/(1-R) versus [NADH]/R, where R is the fractional saturation of the NADH-binding sites (see Gutfreund, 1972). ○, In the absence of Mg²⁺; ●, in the presence of Mg²⁺.

are shown in Fig. 5. In the absence of Mg²⁺ the Scatchard-type replot (Fig. 5b) was not linear [a phenomenon also observed by Hart & Dickinson (1983) and by Pietruszko *et al.* (1985)] and therefore no estimate of the number of sites is possible. In the presence of 20 mM-Mg²⁺ the extrapolation procedure and the replot give a value of 1.9–2.0 for the number of NADH-binding sites (the same as for E-X-CO-NMe₂). This result differs from the previous reports (Bennett *et al.*, 1983; Dickinson & Haywood, 1986) of only approx. 1 NADH-binding site in the presence of Mg²⁺. The reason for the disagreement with the results reported by Bennett *et al.* (1983) is not obvious, but it is worth noting that the titration profile published by Dickinson & Haywood (1986) was obtained with a very high concentration of Mg²⁺ (1 M). With a lower concentration (25 mM) a value for the number of binding sites per molecule of 1.7 was obtained (F. M. Dickinson, personal communication), which is close to the result determined in the present work.

Although aldehyde dehydrogenase is composed of four subunits of identical primary structure (Hempel *et al.*, 1984), it seems that only one subunit reacts with PNP dimethylcarbamate with loss of most of the enzymic

Fig. 5. Fluorescence titration of native aldehyde dehydrogenase in the presence and in the absence of Mg^{2+}

(a) Small volumes of NADH solution were added incrementally to 50 mM-Tris/HCl buffer, pH 7.4, in the presence and in the absence of enzyme, and the fluorescence was determined as described in the Experimental section. $\square$, No enzyme; $\circ$, enzyme ($2.45 \mu\text{M}$); $\bullet$, enzyme ($2.45 \mu\text{M}$) and Mg^{2+} (20 mM). (b) The data in (a) are re-expressed as a plot of $1/(1-R)$ versus $[\text{NADH}]/R$, where R is the fractional saturation of the NADH-binding sites (see Gutfreund, 1972). $\circ$, In the absence of Mg^{2+} ; $\bullet$, in the presence of Mg^{2+} .

activity (Kitson, 1989b) and that it is only this single subunit that can bind NADH (in the absence of Mg^{2+}). A similarly low stoichiometry has been found in, for example, the bursts of NADH or *p*-nitrophenoxide observed when the enzyme acts as a dehydrogenase or as an esterase (Dickinson & Haywood, 1986), and in the inactivation of the enzyme by disulfiram (Dickinson *et al.*, 1981) and by bromoacetophenone (Pietruszko & MacKerell, 1987). According to the present work, a second NADH-binding site becomes available in the presence of Mg^{2+} and the fluorescence of NADH bound at this site can evidently be quenched somewhat by propionaldehyde (see Fig. 1), whereas, as discussed above, NADH bound at the original site in E-X-CO-NMe_2 is immune to fluorescence quenching by propionaldehyde. It is interesting to speculate on how many nucleotide-binding sites might be operative when the native enzyme acts *in vivo*; the concentrations of Mg^{2+} used here are physiologically not unreasonable.

Fig. 6. Rate of displacement of NADH from E-X-CO-NMe_2

At zero time NADH ($3 \mu\text{M}$) was added to E-X-CO-NMe_2 ($1.27 \mu\text{M}$) in 50 mM-sodium phosphate buffer, pH 7.4, at 25°C . After the fluorescence stopped increasing, NAD^+ (1 mM) was added rapidly at the point shown by the small arrow. The inset shows a replot of the fall in fluorescence expressed as a first-order decay process.

Rate of displacement of NADH from $\text{E-X-CO-NMe}_2 \cdot \text{NADH}$

The displacement of NADH from native cytoplasmic aldehyde dehydrogenase on the addition of excess NAD^+ was first studied by MacGibbon *et al.* (1977) by stopped-flow fluorimetry. They observed a biphasic process with rate constants at pH 7.6 of 0.85 and 0.22 s^{-1} . The amplitude of the fast phase was approx. 60% of the total change in fluorescence. Dickinson (1985) reported corresponding values of 3.5 s^{-1} , 0.4 s^{-1} and 35% at pH 7.0, and Blackwell *et al.* (1987) responded with rate constants determined at pH 7.0 of 1.62 and 0.25 s^{-1} . These authors have discussed the discrepancies in their results and the possible bearings that they have on understanding the kinetic mechanism of the enzyme.

Fig. 6 shows the results of experiments with E-X-CO-NMe_2 that are similar in principle to those referred to above with native enzyme. It was found that the dissociation of NADH from E-X-CO-NMe_2 is biphasic with rate constants of 3.4 and 1.8 min^{-1} . We have already seen that the dissociation constant of NADH is approx. 3-fold smaller for the modified form of the enzyme than for the native form; now it is found that the rate of dissociation of NADH is in the region of 15–60 and 7–13 times lower for the modified form than for the native enzyme (for the fast and slow phases of the dissociation respectively, and depending on which of the data referred to above are taken for comparison).

Perhaps the rate of dissociation of NADH from the normal acyl-enzyme (E-X-CO-R) is also very low, as it is from E-X-CO-NMe_2 . If so, one could picture the acyl group and NADH having a kind of co-operative interaction: the acyl group tightens the binding of NADH, ensuring it does not dissociate prematurely, and the NADH in turn may maintain the enzyme in the

correct conformation for catalysis of the hydrolytic step.

It was found that Mg^{2+} lowers the rate of dissociation of NADH from E-X-CO-NMe₂ markedly (results not shown). A control reaction showed that this effect is definitely due to Mg^{2+} and not to the use of Tris buffer in place of phosphate. The rate constants for the biphasic process are 0.47 and 0.37 min⁻¹. A similar slowing was found with native enzyme (Bennett *et al.*, 1983), and it was proposed that this explains the inhibitory effect of Mg^{2+} on the dehydrogenase activity of the enzyme. Again, it seems possible that Mg^{2+} may play some role in regulating the enzyme's activity *in vivo*. It is interesting that relatively minor changes (the presence of Mg^{2+} and of the -CO-NMe₂ label) lower the rate of dissociation of NADH from the enzyme by about two orders of magnitude. Possibly other fairly trivial but as yet unknown factors are responsible for lowering the rate of dissociation of the tightly bound NADH (referred to above) practically to zero.

In summary, the presence of a -CO-NMe₂ label has been shown to have some very major and interesting effects on the interaction of aldehyde dehydrogenase and NADH. Although it is impossible to exclude the idea of 'action at a distance', which is necessary on the supposition that the esterase site is topologically distinct from the dehydrogenase site (Blackwell *et al.*, 1983), it is much easier to envisage PNP dimethylcarbamate as modifying an enzymic nucleophile common to both dehydrogenase and esterase mechanisms, and which necessarily must have a close and intimate relationship with the NADH-binding site. [Preliminary unpublished work involving reaction of the enzyme with [¹⁴C]methyl-labelled PNP dimethylcarbamate, followed by tryptic digestion, has shown that the radioactive label is associated with a peptide identical with residues 297-307 of the human enzyme (von Bahr-Lindström *et al.*, 1984). Although not yet firmly established, it is tempting to conclude that Cys-302 is the reactive nucleophile towards PNP dimethylcarbamate just as it is towards iodoacetamide and a bromoacetyl analogue of NAD⁺ (von Bahr-Lindström *et al.*, 1984; Hempel *et al.*, 1985).]

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REFERENCES

- Bennett, A. F., Buckley, P. D. & Blackwell, L. F. (1982) *Biochemistry* **21**, 4407-4413
- Bennett, A. F., Buckley, P. D. & Blackwell, L. F. (1983) *Biochemistry* **22**, 776-784
- Blackwell, L. F., Bennett, A. F. & Buckley, P. D. (1983) *Biochemistry* **22**, 3784-3791
- Blackwell, L. F., Motion, R. L., MacGibbon, A. K. H., Hardman, M. J. & Buckley, P. D. (1987) *Biochem. J.* **242**, 803-808
- Dalziel, K. (1962) *Biochem. J.* **84**, 240-244
- Dickinson, F. M. (1985) *Biochem. J.* **225**, 159-165
- Dickinson, F. M. (1986) *Biochem. J.* **238**, 75-82
- Dickinson, F. M. & Haywood, G. W. (1986) *Biochem. J.* **233**, 877-883
- Dickinson, F. M., Hart, G. J. & Kitson, T. M. (1981) *Biochem. J.* **199**, 573-579
- Dunn, M. F. & Buckley, P. D. (1985) *Enzymology of Carbonyl Metabolism*, vol. 2: Aldehyde Dehydrogenase, Aldo-Keto Reductase and Alcohol Dehydrogenase (Flynn, T. G. & Weiner, H., eds.), pp. 15-27, Alan R. Liss, New York
- Gutfreund, H. (1972) *Enzymes: Physical Principles*, pp. 68-72, John Wiley and Sons, London
- Hart, G. J. & Dickinson, F. M. (1977) *Biochem. J.* **163**, 261-267
- Hart, G. J. & Dickinson, F. M. (1982) *Biochem. J.* **203**, 617-627
- Hart, G. J. & Dickinson, F. M. (1983) *Biochem. J.* **211**, 363-371
- Hempel, J., von Bahr-Lindström, H. & Jörnvall, H. (1984) *Eur. J. Biochem.* **141**, 21-35
- Hempel, J., Kaiser, R. & Jörnvall, H. (1985) *Eur. J. Biochem.* **153**, 13-28
- Kitson, T. M. (1989a) *Biochem. J.* **257**, 573-578
- Kitson, T. M. (1989b) *Biochem. J.* **257**, 579-584
- MacGibbon, A. K. H., Buckley, P. D. & Blackwell, L. F. (1977) *Biochem. J.* **165**, 455-462
- Motion, R. L., Blackwell, L. F. & Buckley, P. D. (1984) *Biochemistry* **23**, 6851-6857
- Pietruszko, R. & MacKerell, A. D. (1987) *Enzymology and Molecular Biology of Carbonyl Metabolism*, Aldehyde Dehydrogenase, Aldo-Keto Reductase and Alcohol Dehydrogenase (Weiner, H. & Flynn, T. G., eds.), pp. 37-52, Alan R. Liss, New York
- Pietruszko, R., Ferencz-Biro, K. & MacKerell, A. D. (1985) *Enzymology of Carbonyl Metabolism*, vol. 2: Aldehyde Dehydrogenase, Aldo-Keto Reductase and Alcohol Dehydrogenase (Weiner, H. & Flynn, T. G., eds.), pp. 29-41, Alan R. Liss, New York
- von Bahr-Lindström, H., Hempel, J. & Jörnvall, H. (1984) *Eur. J. Biochem.* **141**, 37-42