The Purification and Regulatory Properties of α -Oxoglutarate Dehydrogenase from *Acinetobacter lwoffii*

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The α -oxoglutarate dehydrogenase multienzyme complex was purified from *Acinetobacter lwoffii* to a high degree of homogeneity as shown by gel electrophoresis and analytical ultracentrifugation. Sedimentation-velocity analyses gave $s_{20,w}$ values which increased with increasing protein concentration, suggesting dissociation of the complex in dilute solution. The maximum $s_{20,w}$ value thereby obtained and the value determined by active enzyme centrifugation were both in the range 28–29S. Electron micrographs of the complex indicated a molecular diameter of 20–22 nm (200–220 Å). The overall activity of the complex was inhibited by NADH, and kinetic studies indicated sites of action on the first and third enzyme components. AMP and ADP relieved this inhibition and also stimulated enzyme activity. Assays specific for the first enzyme component showed this to be the site of action of the adenylates. The activity of the complex varied with energy charge in a manner consistent with its role in energy metabolism.

In *Acinetobacter lwoffii* several consecutive enzymes of the tricarboxylic acid cycle have been shown to respond to adenylates (Weitzman & Jones, 1968; Parker & Weitzman, 1970; Weitzman, 1972) and so the flux through the cycle may respond to the energy state of the cell. One of these enzymes is the multi-enzyme complex α -oxoglutarate dehydrogenase, which is inhibited by NADH and stimulated by AMP and ADP, which moreover, relieve the NADH inhibition (Weitzman, 1972).

In an attempt to gain a better understanding of the mechanisms of these complex regulatory responses we have purified the α -oxoglutarate dehydrogenase and examined the behaviour of the overall complex and of its component enzymes. These studies suggest that the first component enzyme of the complex is the major site of action of the regulatory effectors.

Experimental

Materials

Most chemicals were purchased from Sigma (London) Chemical Co. Ltd., Kingston, Surrey, U.K., or from BDH Chemicals Ltd., Poole, Dorset, U.K. The other materials used and their sources were as follows: Sepharose 4B, Pharmacia (Great Britain) Ltd., London W5, U.K.; protamine sulphate, Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; DEAE-cellulose (Whatman DE-11), H. Reeve Angel and Co. Ltd., London EC4, U.K.

Dihydrolipoamide was prepared by electrolytic reduction of a solution containing 1 mM-oxidized lipoamide, 0.1 M-Tris-HCl, pH 8.0, 10 mM-MgCl₂ and 1 mM-EDTA. Electrolysis was performed at 3 V

in the apparatus described by Cecil & Weitzman (1964) and was complete in 1 h, as shown by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959).

The organism used was *Acinetobacter lwoffii*, strain 4B (isolated from a laboratory reservoir of distilled water by P.D.J.W.) and cultures were maintained on nutrient-agar slopes.

Enzyme activities

Spectrophotometric assays. These were done at 25°C with a Unicam SP. 1800 recording spectrophotometer fitted with a thermostatically controlled cuvette holder and results were recorded on a Unicam SP. 21 external recorder. Enzyme activities are expressed in units of μ mol of product formed/min.

The action of effectors on the various enzyme activities was tested by including them in assay mixtures before the addition of enzyme.

α -Oxoglutarate dehydrogenase. The whole enzyme complex was assayed by measuring the formation of NADH at 340 nm. The assay mixture contained 0.1 M-Tris-HCl, pH 8.0, 10 mM-MgCl₂, 1 mM-EDTA, 0.1 mM-thiamin pyrophosphate, 2.5 mM-cysteine hydrochloride, 0.1 mM-CoA, 0.5 mM-NAD⁺ and 1 mM- α -oxoglutarate. The mixtures were incubated for 10 min at 25°C and the reaction was then started by the addition of enzyme solution to give a total volume of 1.0 ml.

When Lineweaver-Burk plots were linear, the values of activity (v) and substrate concentration (A) were fitted to the equation $v = (V_{\max} \cdot A)/(K_m + A)$. Least-squares fits were performed on an Eliot 4100

computer by using FORTRAN computer programs obtained from Professor W. W. Cleland of the University of Wisconsin, Madison, Wis., U.S.A. The programs calculate the most probable values for the apparent kinetic constants $V_{\max}$, and K_m , the slopes and intercepts of the best fit to the primary plots and the standard errors of the estimates. All the standard errors were within 10% of the fitted constants.

α -Oxoglutarate decarboxylase. This component of the enzyme complex was assayed by using a procedure similar to that described by Dietrich & Henning (1970) for the decarboxylase component of pyruvate dehydrogenase by following the oxidative decarboxylation of α -oxoglutarate with dichlorophenol-indophenol as an electron acceptor. The reaction was monitored at 578 nm and the assay mixture contained 0.1 M-potassium phosphate, pH 8.0, 0.5 mM-MgCl₂, 0.1 mM-thiamin pyrophosphate, 1 mM- α -oxoglutarate, 0.05 mM-dichlorophenol-indophenol, 0.5 mg of phenazine methosulphate (stored in the dark) and enzyme solution, in a total volume of 1.0 ml.

Dihydrolipoamide dehydrogenase. This component of the enzyme complex was assayed polarographically with a Radiometer PO4 recording polarograph by using a reaction vessel and procedure as described by Weitzman (1969). The electrodes was a dropping mercury cathode and a saturated calomel reference anode. The enzyme was assayed in the forward direction by following the formation of oxidized lipoamide at -0.75 V with a full-scale recorder deflexion of 0.5 μ A. The assay mixture contained 0.1 M-Tris-HCl, pH 8.0, 10 mM-MgCl₂, 1 mM-EDTA, 0.5 mM-NAD⁺, 0.5 mM-dihydrolipoamide and enzyme solution, in a total volume of 1.0 ml. The solution was deoxygenated by bubbling O₂-free N₂ for 2-3 min and the reaction was then started by the addition of enzyme solution.

Energy charge. 'Energy charge' (Atkinson, 1968) is defined as half the average number of anhydride-bound phosphate groups per adenosine moiety, or

$$\frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

The effects of energy charge on enzyme activity were investigated by incubating appropriate mixtures of AMP and ATP in a buffer of composition 0.1 M-Tris-HCl, pH 8.0, 10 mM-MgCl₂ and 1 mM-EDTA with 10 μ g of adenylate kinase at 37°C for 15 min to reach equilibrium. Portions of these mixtures were then incorporated into the assay mixture to produce the required total adenine nucleotide concentration.

Analytical procedures

Protein determination. Protein concentrations of enzyme extracts up to stage 4 of the purification procedure (DEAE-cellulose chromatography) were determined by the Folin method (Lowry *et al.*, 1951)

with bovine serum albumin (Sigma) as a standard. After stage 4, protein concentrations were determined from the absorbance values at 260 nm and 280 nm (Warburg & Christian, 1941).

Analytical polyacrylamide-disc-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed by the method of Davis (1964). Gels were prepared in 10 cm $\times$ 0.5 cm glass tubes. The sample (40-50 μ g of protein), in a volume not exceeding 0.1 ml, was made 10% (w/v) in sucrose and coloured with 0.001% Bromophenol Blue. Electrophoresis was carried out at 2 mA/gel, after which the gels were stained for protein or α -oxoglutarate dehydrogenase activity.

Protein was stained with Coomassie Brilliant Blue (Chrambach *et al.*, 1967). The gels were first fixed in 12.5% (w/v) trichloroacetic acid for 30 min and then stained in 1% (w/v) Coomassie Brilliant Blue in 12.5% trichloroacetic acid for 60 min. The gels were stored in 10% (w/v) trichloroacetic acid.

α -Oxoglutarate dehydrogenase activity was detected by incubating the gels in the dark at 37°C for 10 min in a solution containing 0.1 M-Tris-HCl, pH 8.0, 10 mM-MgCl₂, 1 mM-EDTA, 0.1 mM-thiamin pyrophosphate, 5 mM- α -oxoglutarate, 0.1 mM-CoA, 0.5 mM-NAD⁺, 0.5 mM-AMP, 1 mg of phenazine methosulphate and 3 mg of Nitro Blue Tetrazolium, in a total volume of 10 ml.

Analytical ultracentrifugation. Sedimentation-velocity analyses were performed in an MSE analytical ultracentrifuge equipped with a u.v. scanner. Samples were sedimented at 55000 rev./min at 20°C and scanned at known time-intervals at 280 nm. Boundary positions (r_b) were estimated from the first-derivative curves of the scanner print-out. The sedimentation coefficients were computed on a Hewlett Packard 9100B calculator from a linear regression analysis of $\log_e r_b$ against time, by using a program (S-1) kindly provided by Dr. A. J. Rowe of this Department, and were corrected to $s_{20,w}$ values by making allowance for the temperature-correction factors of the buffer solution (Bowen, 1970b).

Active enzyme centrifugation was performed as described by Cohen & Mire (1971a,b). Samples of α -oxoglutarate dehydrogenase (0.5-1.0 μ g of protein) were sedimented at 40000 rev./min in a Vinograd cell (MSE) through substrate solution at 20°C containing 60 mM-Tris-HCl, pH 8.0, 10 mM-MgCl₂, 1 mM-EDTA, 10% (v/v) glycerol, 0.5 mM-AMP, 0.1 mM-thiamin pyrophosphate, 3 mM-cysteine hydrochloride, 2 mM-NAD⁺, 6 mM- α -oxoglutarate and 0.5 mM-CoA, and the formation of NADH was monitored at 340 nm. The method requires that every enzyme molecule should react under identical conditions. This condition was satisfied by using saturating substrate concentrations (approx. $30 \times K_m$) and by including AMP to overcome product inhibition by NADH. The sedimentation coefficient was computed from boundary positions as described above.

Electron microscopy

Samples of α -oxoglutarate dehydrogenase complex were first fixed in buffer of composition 20mM-Tris-HCl, pH8.0, 10mM-MgCl₂, 1mM-EDTA and 0.5% (v/v) glutaraldehyde for 12–16h to prevent disruption of the complex on the grid. Drops of the fixed enzyme solution (0.1mg of protein/ml) were placed on porous carbon-coated copper grids (400 mesh) for approx. 30s. Excess of protein was removed by washing with a drop of water and the sample was then negatively stained with a drop of 2% (w/v) uranyl acetate for 1min. After drying, the specimen grids were examined in a Siemens Elmiskop 1A electron microscope at an accelerating voltage of 80kV and a magnification of $\times 40000$.

Results

Purification and characterization of the α -oxoglutarate dehydrogenase complex

Throughout the purification, buffer of composition 20mM-Tris-HCl, pH8.0, 10mM-MgCl₂ and 1mM-EDTA was used and, in this section, is referred to as 'MET-8 buffer'.

Stage 1: preparation of cell-free extract. *A. lwoffii* was grown in 15 litres of 2% (w/v) nutrient broth (Oxoid) at 30°C with vigorous aeration and harvested in stationary phase with a Sharples continuous-flow centrifuge. The wet paste of cells was washed in MET-8 buffer and stored frozen at -4°C, under which conditions α -oxoglutarate dehydrogenase activity remained stable for several weeks.

The cells were disrupted by suspending them in cold MET-8 buffer at $E_{680} = 100$ and passing them through a pre-cooled French pressure cell at 8.3MPa (12000lb/in²). Cell debris and unbroken cells were removed by centrifugation at 11000g ($r_{av.} = 9.8$ cm) for 2h at 4°C. All subsequent purification steps were performed at 4°C.

Stage 2: protamine sulphate fractionation. The cell-

free extract was diluted to a protein concentration of 20mg/ml with MET-8 buffer. A 2% (w/v) aqueous solution of protamine sulphate was added dropwise with continuous stirring to a final concentration of 0.9mg/10mg of protein and the mixture was left to equilibrate for 30min. The precipitate was removed by centrifugation at 11000g for 10min and discarded.

Stage 3: isoelectric precipitation. The supernatant solution obtained above was acidified to pH5.3 with acetic acid (7%, v/v) and left for 30min. The precipitate was removed by centrifugation at 11000g for 10min and discarded. The supernatant solution was then adjusted to pH4.8 with acetic acid and left for 30min. After centrifugation as described above the precipitate was dissolved in 10ml of MET-8 buffer containing 20% (v/v) glycerol and dialysed against 500ml of this buffer for 12–16h.

Stage 4: DEAE-cellulose chromatography. The dialysed enzyme solution was applied to a column (40cm $\times$ 2.5cm) of DEAE-cellulose equilibrated with MET-8 buffer containing 20% glycerol. The enzyme was then washed into the column with approx. 200ml of this MET-8–20% glycerol buffer containing 0.15M-KCl, and a linear salt gradient of 0.15–0.40M-KCl in a total volume of 700ml of the same buffer was then applied. Fractions (5ml) were collected and assayed for enzyme activity and protein. The enzyme was eluted when the salt concentration was approx. 0.23M and those fractions containing enzyme at a specific activity greater than 8 units/mg of protein were combined.

Stage 5: ammonium sulphate fractionation. Ammonium sulphate (31.3g/100ml) was added slowly to the combined fractions and left for 30min. After centrifugation at 11000g the precipitate was dissolved in 2ml of MET-8–20% glycerol buffer.

Stage 6: gel filtration on Sepharose 4B. The concentrated enzyme solution was applied to a column (40cm $\times$ 2.5cm) of Sepharose 4B equilibrated with MET-8 buffer and eluted at approx. 10ml/h with this buffer. Fractions (2ml) were collected and assayed

Table 1. Purification of α -oxoglutarate dehydrogenase

The purification was carried out as described in the text, starting with 15 litres of stationary-phase culture of *A. lwoffii* grown on nutrient broth.

Stage of purification	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)
(1) Crude extract	140	600	2290	0.26	100
(2) Protamine sulphate	144	546	1370	0.40	91
(3) Isoelectric precipitation	11	354	320	1.1	59
(4) DEAE-cellulose	35	150	13	11.5	25
(5) Ammonium sulphate	2	114	10	11.4	19
(6) Sepharose 4B	14	65	3.6	18.0	11
(7) Ammonium sulphate	2.5	46	2.2	21.0	8

for enzyme activity and protein. α -Oxoglutarate dehydrogenase was eluted as a symmetrical peak with complete coincidence of protein and enzyme activity.

The enzyme was concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (31.3 g/100 ml), suspended in 2 ml of MET-8–20% glycerol buffer and dialysed against 500 ml of this buffer. The α -oxoglutarate dehydrogenase activity was stable at 4°C for several weeks. A summary of the purification is presented in Table 1.

Analytical disc-gel electrophoresis. α -Oxoglutarate dehydrogenase was the major protein band, with a mobility of 0.07 relative to the Bromophenol Blue marker, when electrophoresis was carried out at pH 8.9 and the gels were stained for protein and enzyme. Two minor bands, with mobilities of approx. 0.65 and 0.7, were also observed on staining for protein but showed no activity in the enzyme stain.

Since the pH of polyacrylamide gels may increase by approx. 1 unit during electrophoresis (Orr *et al.*, 1972), and alkaline conditions are known to bring about dissociation of the α -oxoglutarate dehydrogenase complex from *Escherichia coli* (Mukherjee *et al.*, 1965), it is possible that the minor bands observed arose from dissociation of the complex. Attempts to investigate this possibility by carrying out electrophoresis at pH 7.0 were unsuccessful insofar as protein was detected only at the top of the gel.

Sedimentation-velocity analysis. When the purified enzyme was examined in the analytical ultracentrifuge the absorbance at 280 nm and the derivative curve were recorded. The total change of protein concentration over the boundary remained constant except for a small change due to radial dilution. As a consequence, the derivative curve was a single symmetrical peak, indicating that the enzyme was essentially pure. The sedimentation coefficient was

Table 2. Dependence of $s_{20,w}$ on protein concentration

Samples of *A. lwoffii* α -oxoglutarate dehydrogenase in buffer of composition 20 mM-Tris-HCl, pH 8.0, 10 mM-MgCl₂, 1 mM-EDTA and 20% (v/v) glycerol were sedimented at 55000 rev./min at 20°C in an MSE analytical ultracentrifuge and scanned at known time-intervals at 280 nm. Sedimentation coefficients were computed and corrected to $s_{20,w}$ values as described in the Experimental section.

Concn. of protein (mg/ml)	$s_{20,w}$
0.21	21.2
0.25	22.3
0.43	25.5
0.60	26.1
0.82	28.1

determined as described in the Experimental section and shown to have an unusual dependence on protein concentration (Table 2). The sedimentation coefficient increased in value with increasing protein concentration, in contrast with the behaviour of most proteins (Bowen, 1970a). These results are consistent with an extremely rapid equilibration between the

Fig. 1. Band sedimentation pattern of active α -oxoglutarate dehydrogenase

Sedimentation of active α -oxoglutarate dehydrogenase was monitored by absorption optics at 340 nm. Enzyme solution (15 μ l; 60 μ g/ml) was layered on 0.30 ml of substrate solution (see the Experimental section). The trace was obtained 10 min after reaching 40050 rev./min and sedimentation is from right to left. R_1 and R_2 are reference slits. a is the absorption due to the appearance of NADH, b is the beginning of the cell and p and q represent the derivatives of a and b respectively.

subunits and the whole complex, in which the equilibrium favours the formation of the latter. It was not possible to obtain the purified enzyme at concentrations greater than 0.8mg/ml and therefore the sedimentation coefficient of 28S obtained at this enzyme concentration was assumed to be a minimum value.

α -Oxoglutarate dehydrogenase purified through stage 4 (DEAE-cellulose chromatography) was also investigated by active enzyme centrifugation. The 340nm scan and the first-derivative pattern were as shown in Fig. 1 and the sedimentation coefficient was determined to be in the range 28–29S. It appears therefore that the binding of substrates to the enzyme complex prevents dissociation even at the low protein concentrations used in active enzyme centrifugation.

Electron microscopy. Samples of α -oxoglutarate dehydrogenase negatively stained with uranyl acetate were examined in the electron microscope. The enzyme complex is a polyhedron of approximate diameter 20–22nm (200–220Å) and there appear to be 'holes' in the centre of many of the enzyme molecules.

Effect of pH on enzyme activity. The dependence of enzyme activity on pH was determined at 1mM- α -oxoglutarate in the absence and presence of 0.2mM-AMP in 0.1M-Tris-HCl buffers ranging from pH 6.5 to 9.5 (Fig. 2). A true pH optimum could not be obtained as it was not possible to saturate the enzyme with α -oxoglutarate, substrate inhibition being marked particularly at concentrations above 10mM- α -

Fig. 2. Dependence of α -oxoglutarate dehydrogenase activity on pH

Assays were carried out under standard conditions in 0.1M-Tris-HCl buffers at various pH values. ○, Enzyme alone; ●, enzyme with 0.2mM-AMP.

oxoglutarate. The dependence of enzyme activity on pH in the absence of AMP essentially reflects an alteration of the apparent K_m for α -oxoglutarate. The apparent K_m increased with pH, being 7.1mM at pH 9.0, 2.4mM at pH 8.0 and 0.8mM at pH 7.0. In the presence of AMP, when α -oxoglutarate is present at near-saturating concentrations, there is a broad pH optimum for enzyme activity.

Regulatory properties of the α -oxoglutarate dehydrogenase complex

Effect of adenine nucleotides. The effects of AMP and ADP on the purified α -oxoglutarate dehydrogenase complex, the α -oxoglutarate decarboxylase component (E1) and the dihydrolipoamide dehydrogenase component (E3) were investigated. Fig. 3 shows the dependences of activation on effector concentration for the complex and component E1 when assays were carried out at 1mM- α -oxoglutarate. The activation was essentially maximal at AMP and ADP concentrations of 0.1mM and concentrations of AMP as low as 0.01mM produced considerable stimulation. In contrast, both nucleotides were completely without effect on component E3.

The effects of AMP and ADP on the kinetic characteristics of the enzyme complex and component E1 were then examined. The effect of 0.2mM-adenine

Fig. 3. Dependence of activation of the α -oxoglutarate dehydrogenase complex and the α -oxoglutarate decarboxylase (E1) component on adenine nucleotide concentration

Enzyme activities were measured under standard conditions (see the Experimental section) in the absence and presence of 0.2mM-AMP or 0.2mM-ADP, and the percentage activation was thereby determined. ○, Whole complex with AMP; ●, whole complex with ADP; □, component E1 with AMP; ■, component E1 with ADP.

nucleotide on the rate dependences of the enzyme activities on α -oxoglutarate concentration is shown in Fig. 4. In both cases the stimulation resulted from a marked decrease in the apparent K_m for α -oxoglutarate.

Fig. 4. Effect of adenine nucleotides on the dependence of enzyme activity on α -oxoglutarate concentration

Enzyme activity was measured at 0.5 mM-NAD⁺ and 0.1 mM-CoA in the absence of adenylates (●), or in the presence of 0.2 mM-AMP (Δ) or 0.2 mM-ADP (○). (a) α -Oxoglutarate dehydrogenase complex; (b) α -oxoglutarate decarboxylase (component E1).

Fig. 5. Dependence of enzyme inhibition on NADH concentration

The effect of NADH on α -oxoglutarate dehydrogenase activity was measured at 5 mM- α -oxoglutarate, 0.5 mM-NAD⁺ and 0.1 mM-CoA, and that on dihydro-lipoamide dehydrogenase was measured under standard assay conditions. ○, α -Oxoglutarate dehydrogenase complex; ●, dihydro-lipoamide dehydrogenase (component E3).

Effect of NADH. Both the whole enzyme complex and component E3 were inhibited by low concentrations of NADH (Fig. 5). It should be noted that in the assay of component E3, NADH concentrations as high as 3 mM were investigated, a condition which is impossible in the normal spectrophotometric assay of this enzyme and of the whole enzyme complex. The polarographic assay monitors oxidized lipoamide at a selected potential at which reduced lipoamide, NAD⁺ and NADH do not interfere and therefore allows any concentration of NADH to be examined.

The effect of 0.1 mM-NADH on the rate-dependence of component E3 on NAD⁺ was examined at 0.5 mM-dihydrolipoamide. The apparent K_m for NAD⁺ is 0.20 mM. NADH is a competitive inhibitor of component E3 with respect to NAD⁺ with $K_i = 45 \mu\text{M}$.

The adenine nucleotides were without effect on the NADH inhibition of component E3. This result contrasts with the relief of NADH inhibition by AMP and ADP observed with the whole complex, suggesting an additional site of action for NADH. The effect of NADH on component E1 could not be examined directly because of the non-enzymic reduction of dichlorophenol-indophenol by NADH. Nevertheless indirect evidence for an additional site of action for NADH has been obtained from studies of the inhibition of the whole enzyme complex.

The effect of NADH on the rate dependence of whole enzyme complex on α -oxoglutarate concentration was determined at non-saturating (0.2 mM) and near-saturating (2 mM) concentrations of NAD⁺. Fig. 6 shows that when assays were carried out at 0.2 mM-NAD⁺ the percentage inhibition decreased as

Fig. 6. Effect of NADH on the dependence of α -oxoglutarate dehydrogenase activity on α -oxoglutarate

(a) Enzyme activity was measured at 0.2 mM-NAD⁺ and 0.1 mM-CoA in the absence (○) and the presence (●) of 0.09 mM-NADH. (b) Enzyme activity was measured at 2.0 mM-NAD⁺ and 0.1 mM-CoA in the absence (○) and the presence (●) of 0.09 mM-NADH.

Fig. 7. Joint effects of NADH and AMP on enzyme activity

Assays were carried out as described in the text in the presence of 1 mM- α -oxoglutarate and 0.1 mM-NADH. NAD⁺ concentrations were: $\circ$, 0.1 mM; $\bullet$, 0.5 mM; Δ , 2.5 mM.

the concentration of α -oxoglutarate was increased, although extrapolation to infinite α -oxoglutarate concentration indicated that the NADH inhibition was not completely overcome. However, when the NAD⁺ concentration was increased to 2 mM the inhibition by NADH appeared to be strictly competitive with respect to α -oxoglutarate. The effects of NADH on the rate dependence of the whole complex on NAD⁺ concentration, determined at non-saturating (1 mM) and near-saturating (5 mM) concentrations of α -oxoglutarate, were similar. In both cases the percentage inhibition decreased as the concentration of NAD⁺ was increased, but only at the high concentration (5 mM) of α -oxoglutarate did the double-reciprocal plots intersect at infinite NAD⁺ concentration. These results suggest that NADH inhibits component E1 in competition with α -oxoglutarate and component E3 in competition with NAD⁺.

Joint effects of NADH and AMP. AMP both overcomes NADH inhibition of α -oxoglutarate dehydrogenase and stimulates enzymic activity. The present study shows that the extent of AMP stimulation in the presence of NADH relative to that in its absence depends on the NAD⁺ concentration.

Assays were first carried out at 1 mM- α -oxoglutarate and 0.1 mM-NAD⁺. Enzyme activity measured in the presence of 0.2 mM-AMP was assigned as maximum activity. Assays were then carried out in the presence of 0.1 mM-NADH with various concentrations of AMP and the dependence of the percentage of maximum activity was plotted against AMP concentra-

Fig. 8. Dependence of enzyme activity on energy charge

The effect of energy charge on α -oxoglutarate dehydrogenase activity was examined at total adenylate concentrations of 0.2 mM ($\bullet$) and 1 mM ($\circ$) in the presence of 1 mM- α -oxoglutarate and 0.1 mM-NADH. 'Energy charge' is defined as the ratio $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$.

tion. Similar dependences were obtained at 0.5 mM- and 2.5 mM-NAD⁺.

Fig. 7 shows that the extent to which AMP stimulates enzyme activity in the presence of NADH relative to that in its absence increases with increasing NAD⁺ concentration. These results lend support to the suggestion that NADH inhibits the enzyme at two sites and that AMP overcomes the inhibition of component E1 and NAD⁺ overcomes the inhibition of component E3.

The relief of NADH inhibition by ADP was similar but a higher concentration was required.

Effect of energy charge. The effect of varying energy charge at a constant total adenine nucleotide concentration was examined by introducing equilibrium mixtures of AMP+ADP+ATP into the enzyme assay. The assays were carried out with 1 mM- α -oxoglutarate, and 0.1 mM-NADH was included so that both inhibitory and stimulatory effects would be exerted simultaneously and thereby better simulate the likely physiological condition in the cell. Fig. 8 shows that at total adenylate concentrations of 0.2 mM and 1 mM the enzyme is fully active over the energy charge range 0–0.7, but activity decreases steeply above this value.

Discussion

The initial observation of the regulatory properties of α -oxoglutarate dehydrogenase from *A. lwoffii*

(Weitzman, 1972) prompted us to examine this system in greater detail. We have purified the enzyme and investigated both the activities and regulatory properties of the whole complex and of the first and third component enzymes.

The sedimentation coefficient of the *A. lwoffii* α -oxoglutarate dehydrogenase complex determined by active enzyme centrifugation was 28–29S. Values determined for the sedimentation coefficient of the pig heart enzyme complex range from 31.5 to 36.0S (Hirashima *et al.*, 1967; Tanaka *et al.*, 1972) whereas that for the *Escherichia coli* enzyme has been reported to be 40S (Koike *et al.*, 1960). The difference between the sedimentation coefficients of the *A. lwoffii* and pig heart complexes is consistent with the difference in molecular size measured by electron microscopy. Our electron micrographs of the *A. lwoffii* complex show it to be a polyhedron of diameter 20–22nm (200–220 Å), compared with a diameter of approx. 26nm (260 Å) reported for the pig heart enzyme (Tanaka *et al.*, 1972). Electron-microscopic studies of the *E. coli* complex (Reed & Oliver, 1968) have been hindered by its dissociation during negative staining, but published micrographs indicate a diameter similar to that of the *A. lwoffii* enzyme.

The regulatory properties previously observed with unpurified preparations of *A. lwoffii* α -oxoglutarate dehydrogenase (Weitzman, 1972) have been fully confirmed with the purified enzyme. These are: (i) inhibition of the enzyme by NADH; (ii) relief of the NADH inhibition by AMP or ADP; (iii) stimulation of the enzyme by AMP or ADP.

It is possible to assay specifically the first and third components (E1 and E3) within the complex, although we have not found a satisfactory assay for the second component (E2). We have shown component E1 to be the site of action of the adenylate effectors, AMP and ADP producing a stimulation of component E1 activity and a decrease in the apparent K_m for α -oxoglutarate in a manner resembling that for the whole complex.

In *E. coli* pyruvate dehydrogenase, component E1 has been shown to be regulated by the effectors fructose 1,6-diphosphate, phosphoenolpyruvate and acetyl-CoA and it has been suggested that this component is the most suitable site of action because it catalyses the first, and apparently irreversible, step in the reaction sequence (Schwartz *et al.*, 1968; Shen & Atkinson, 1970).

The adenine nucleotide regulation of α -oxoglutarate dehydrogenase in higher organisms has recently been reported. In cauliflower florets the enzyme is stimulated by AMP with a decrease in the apparent K_m for α -oxoglutarate (Wedding & Black, 1971) similar to that observed for the *A. lwoffii* enzyme. The stimulation has been found to be exerted on the E1–E2 sub-complex.

In blowfly flight muscle, ATP inhibition of the

enzyme has been demonstrated and the inhibition is reported to be overcome by AMP and ADP (Hansford, 1972). The site of action of the adenine nucleotides within the enzyme complex has not been identified, although the competitive inhibition by ATP with respect to α -oxoglutarate would suggest component E1. However, the ferricyanide-linked assay did not fully confirm this suggestion, as the ATP inhibition was found to be greatly decreased.

It was not possible to identify unambiguously the sites of action of NADH on the *A. lwoffii* complex. One site of NADH inhibition was identified as component E3 but it was experimentally impossible to test the effect of NADH on component E1. Evidence that NADH may indeed also act on component E1 has therefore been gained indirectly. When the slopes of double-reciprocal plots, obtained at non-saturating concentrations of NAD⁺ or α -oxoglutarate in the presence of various NADH concentrations, were re-plotted against the NADH concentration the shape of the curves was parabolic (Parker, 1972). This indicated that NADH binds at two sites on the enzyme (Cleland, 1970). The competitive effects between NADH and both α -oxoglutarate and the adenylates point to component E1 as being the second site. It appears therefore that whereas the adenylates act only on component E1 NADH acts on components E1 and E3.

NADH inhibition of the *E. coli* α -oxoglutarate dehydrogenase has also been observed (Hansen & Henning, 1966) and the inhibition is competitive with respect to NAD⁺, indicating that the likely site of action is component E3 of the complex. In rat liver the enzyme is reported to be inhibited by both NADH and succinyl-CoA (Garland, 1964). Inhibition of the *A. lwoffii* enzyme by succinyl-CoA occurs only at extremely high, unphysiological, concentrations.

When enzymes are affected by more than one adenine nucleotide it may be useful to examine enzyme activity as a function of energy charge (Atkinson, 1968). Recently the energy charge in *E. coli* has been estimated during growth and starvation and it has been concluded that growth can occur only at energy-charge values above 0.8 (Chapman *et al.*, 1971). In addition, various data on the nucleotide concentrations in cells were drawn together and values for the energy charge calculated. Most of these values were in the range 0.75–0.90.

α -Oxoglutarate dehydrogenase exhibited maximum activity over a wide range of energy charge (0–0.7), with a sharp decrease at 0.7–0.9. Such a response is similar to that predicted by Atkinson (1968) for enzymes involved, as is this one, in ATP-regenerating systems.

As was pointed out by Weitzman (1972), the regulation of the α -oxoglutarate dehydrogenase in *A. lwoffii* may be essential for the control of the tricarboxylic acid cycle or a particular segment of it. The

operation of the cycle leads to the regeneration of ATP and it might be expected that control would be exerted by the energy state of the cell. The adenylate effects on α -oxoglutarate dehydrogenase would serve to stimulate the activity of the cycle, resulting ultimately in phosphorylation and a decrease in the AMP and ADP concentrations in the cell.

α -Oxoglutarate is a branch-point metabolite capable of being metabolized either by α -oxoglutarate dehydrogenase or by glutamate dehydrogenase. The fate of such metabolites depends largely on the relative substrate affinities of the competing enzymes. The dual control of the *A. lwoffii* α -oxoglutarate dehydrogenase by the low-energy signals AMP and ADP and by NADH, the end-product of both the cycle and the complex itself, may permit sensitive control of the branch-point. There is a marked similarity between the feedback inhibition of enzyme complexes and of biosynthetic pathways whose first enzyme is subject to end-product inhibition. Indeed, the reactions catalysed by α -oxo acid dehydrogenase multienzyme complexes constitute short metabolic pathways.

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