INACTIVATION OF PURIFIED OX KIDNEY BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEX BY PHOSPHORYLATION

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1. Introduction

BCDH complex in extracts of rat heart, kidney, liver and skeletal muscle mitochondria is inactivated by ATP [1–5]. Inactivation by ATP is inhibited by ketoleucine, reversed by PDHP phosphatase preparations [1,3,5] and correlated with incorporation of ^{32}P from [γ-^{32}P]ATP into a protein of M_r 48 000 assumed to be the α-chain of BCDH [5–7]. Inactivation, ^{32}P incorporation and reactivation has been shown in mitochondria [1,3,5–7,9] and interconversion has been shown in perfused rat heart [3].

Further progress is hampered by the lack of a bulk source of purified BCDH complex inactivated by ATP. Ox kidney mitochondria is the most convenient bulk source but in [8] no inactivation of ox kidney complex by ATP was found. Although we have confirmed these findings with procedure A in [8] it seemed important to reinvestigate the ox kidney complex using different methods of purification. We describe here phosphorylation and inactivation by ATP of purified ox kidney BCDH complex free of PDH complex.

2. Experimental

Sources of materials were as in [5,10,11]. BCDH complex was assayed spectrophotometrically [5] on samples containing 5–10 munits (prior to ATP inactivation). PDH complex was assayed likewise with 1 mM pyruvate. Protein bound ^{32}P was assayed on paper squares [11]. SDS–PAGE and autoradiography of gels was as in [5]. Buffers: (A) 30 mM potassium phosphate/5 mM DTT/5 mM EGTA/0.2 mM TPP/1% (v/v) ox serum/5% (v/v) Triton/pH 7.5; (B) as (A) omitting Triton but with 0.25 M NaCl. For ATP inactivation $MgCl_2$ (to 10 mM), oligomycin B (to 25 μg/ml) and ATP (to 1 mM unless given) were added; incubations were at 30°C with controls lacking ATP.

Ox kidney cortex mitochondria were isolated from an homogenate (Atomix homogeniser 4 × 15 s bursts) in 0.25 M sucrose/5 mM Tris–HCl/2 mM EGTA/pH 7.5, by centrifugation at 800 × *g* for 20 min (to remove debris) and 31 800 × *g* for 20 min (to pack mitochondria). The 800 × *g* pellet was re-extracted once. Mitochondria were resuspended and repelleted in sucrose medium 3×. Extracts were prepared by freezing (liquid N_2) and thawing (30°C) 3× in buffer A (50 mg protein/ml) and clarified at 49 600 × *g* for 15 min.

Abbreviations: BCDH, branched chain 2-oxoacid dehydrogenase; DTT, dithiothreitol; EGTA, ethanedioxybis(ethylamine)-tetraacetate; ketoleucine, 4-methyl 2-oxopentanoate; MOPS, 2-(*N*-morpholino)propane sulphonate; PEG, polyethylene glycol; PDH, pyruvate dehydrogenase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TPP, thiamin pyrophosphate

3. Results and discussion

The objective was to prepare BCDH complex free of PDH complex that was inactivated by phosphorylation with ATP. Criteria of purification were: activity ratio (BCDH complex/PDH complex); absence of PDH complex subunits on SDS–PAGE; absence of ^{32}P in α-subunit of PDH on SDS–PAGE; protein was not assayed. Purified BCDH complex free of PDH complex oxidises pyruvate [8]; the expected activity ratio was 6:1. M_r-Values for the subunits of the bovine kidney BCDH and PDH complexes are given in [8,12]. The M_r of the BCDH α-subunit is 46 000 and

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that of the PDH α-subunit 41 000. In what follows, results of ATP inactivation and of controls are given as the % change in BCDH activity/time of incubation, i.e., a negative change is loss of activity. Results are means of 2 obs., which on average differed by <6% of their mean.

3.1. *Pilot experiments*

BCDH complex in mitochondrial extracts was inactivated by 0.5 mM ATP (ATP, −32%/15 min; control ±0%). The results differ from those in [8] (no inactivation) perhaps because ATP hydrolysis was minimised by oligomycin B and low protein concentration (1 mg/ml) [5,6]. After precipitation by centrifugation at 157 000 × *g* for 2 h and solution in buffer B inactivation by ATP was retained (ATP, −80%/30 min; control +4%) and was associated with incorporation of ^{32}P from [γ-^{32}P]ATP into the α-chain of BCDH (fig.1A). Ketoleucine inhibited inactivation (not given) and incorporation (fig.1B). BCDH complex precipitated with 5% PEG at pH 6.4 and taken up in modified buffer B (50 mM MOPS (pH 7) in place of phosphate) was not inactivated by ATP (ATP, −11%/30 min; control −14%). MOPS buffer inhibited inactivation. Dilution by 10-fold with buffer B restored

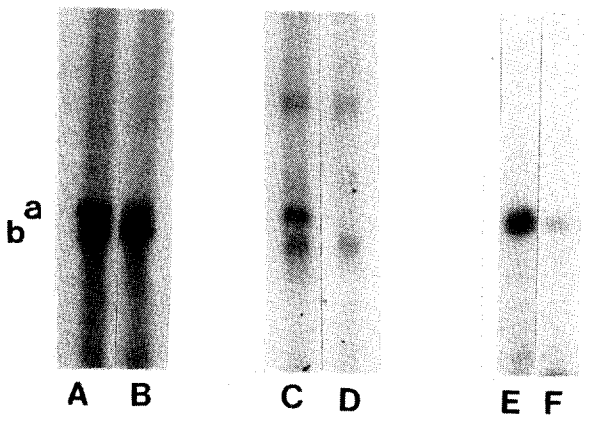

Fig.1. Autoradiographs of gels after SDS–PAGE of preparations of BCDH complex incubated with [γ-^{32}P]ATP: (A,C,E) ATP alone; (B,D,F) with 2 mM ketoleucine + ATP; (A,B) 157 000 × *g* pellet from mitochondrial extracts taken up in buffer B; BCDH complex, 0.90 units/ml; 1 mM [γ-^{32}P]ATP, 440 dpm/pmol. (a) M_r 462 000 (BCDHα); (b) M_r 42 200 (PDHα). (C,D) 157 000 × *g* pellet from fraction obtained with 2% PEG at pH 7.5; BCDH complex, 0.56 units/ml; 0.3 mM [γ-^{32}P]ATP; 2300 dpm/pmol. (E,F) Fraction obtained by precipitation at pH 6.4; BCDH complex, 1.0 units/ml; 0.3 mM [γ-^{32}P]ATP; 2300 dpm/pmol.

inactivation (ATP, −91%/30 min; control ±0%); 10-fold dilution in MOPS buffer B did not (ATP, −11%/30 min; control +7%). Chromatography on hydroxyapatite [7] was unsuccessful as separation from PDH complex was incomplete and ATP inactivation was lost. Precipitation of BCDH complex at pH 6.2 [8] led to loss of inactivation by ATP. It was then discovered that omission of TPP and ox serum from buffer B resulted in precipitation of BCDH complex, at pH 6.4; the preparation was inactivated by ATP and apparently free of PDH complex. The protocol of two successful preparations was as follows; all operations were at 4°C.

3.2. *Purification of BCDH complex*

Fresh ox kidney cortex (650 g) gave 25 ± 2 g mitochondrial protein and mitochondrial extract contained 96 ± 6.5 units of BCDH complex (mean ± SEM for 7 obs.). The activity ratio (BCDH complex/PDH complex) was <0.07 (because of lactate dehydrogenase, PDH complex is under-estimated). $MgCl_2$ was added to 10 mM and BCDH complex precipitated at pH 7.5 with 0.04 vol. 50% (w/v) PEG, and separated at 49 600 × *g* for 15 min, taken up in buffer B, and clarified after 60 min by centrifugation. Recovery of BCDH complex was 59 ± 5.3 (6 obs.); activity ratio was 2.7 ± 0.23 (6 obs.); the complex was inactivated by ATP (ATP, −39%/20 min; control −7%). The solution was centrifuged twice at 100 000 × *g* for 15 min; this removed brown pigmented material. The recovery of BCDH complex (supernatant) was 75 ± 5% (4 obs.) the activity ratio was 3.0 ± 0.25 (4 obs.).

The BCDH complex was precipitated and separated from proteins of lower M_r (including lactate dehydrogenase) by centrifugation at 157 000 × *g* for 2 h, taken up in phosphate/DTT/EGTA/pH 7.5 and clarified by centrifugation (100 000 × *g*/10 min). The recovery of BCDH complex (supernatant) was 76%; the activity ratio was 2.9 ± 0.9 (4 obs.). BCDH complex was inactivated by ATP (ATP, −100%/10 min; control ±0%). With [γ-^{32}P]ATP, ^{32}P was incorporated into the α-chains of BCDH and PDH (fig.1C). Ketoleucine inhibited ^{32}P incorporation into the BCDH α-chain (fig.1D) and inactivation (not given). SDS–PAGE showed subunits of both complexes (not given). The solution was adjusted to pH 6.8, insoluble material removed and discarded after 10 min (49 600 × *g*/10 min), and the pH of the supernatant adjusted to 6.4. After 10 min the precipitate was collected (49 600 × *g*/10 min) and taken up in phosphate/DTT/

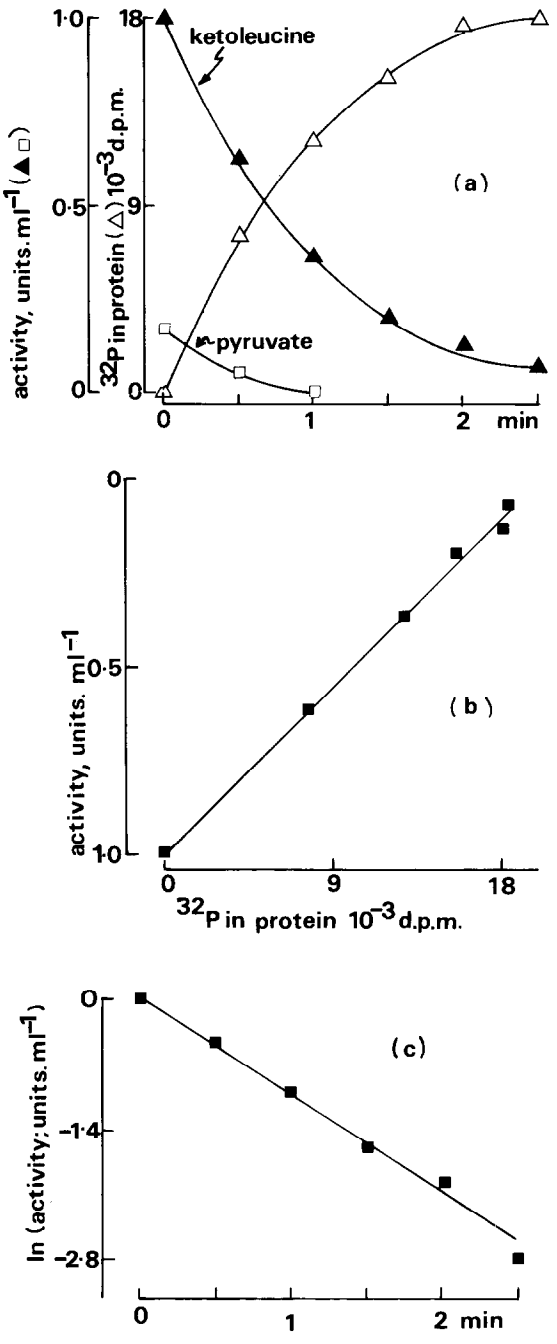

Fig.2. Inactivation and incorporation of ^{32}P into purified ox kidney BCDH complex (1 unit/ml) incubated at 30°C with [γ-^{32}P]ATP (0.3 mM; 323 dpm/pmol). At the times shown in (a) 25 μl samples were taken for assay of ^{32}P in protein and two 5 μl samples for assay of BCDH complex activity with ketoleucine and pyruvate. Controls lacking ATP showed no change in activities (not given). Each point is mean of 2 obs. Note inverted ordinate in (b).

EGTA/pH 7.5. The recovery of BCDH complex was 23% (overall yield 6%); the activity ratio (ketoleucine/pyruvate) was 6.0–7.9. SDS–PAGE showed the 3 subunits of the BCDH complex together with low M_r material near the dye front; subunits of the PDH complex were absent (not given).

3.3. *Phosphorylation and inactivation of purified BCDH complex*

Pilot experiments showed inactivation of BCDH complex by 0.3 mM ATP (ATP, −97%/3 min, −100%/6 min; control −8%, −5%).

SDS–PAGE after incubation for 20 min with 0.3 mM [γ-^{32}P]ATP showed a single band of ^{32}P corresponding to the α-chain of BCDH; this incorporation was inhibited by 2 mM ketoleucine (fig.1E,F). Fig.2(a) shows the time course of inactivation and of ^{32}P incorporation into protein. The pyruvate dehydrogenase activity (assumed to be due to oxidation of pyruvate by BCDH complex) was lost concurrently with BCDH complex activity. Inactivation of BCDH complex was 94% in 2.5 min; activity with pyruvate fell to the limit of detection in 1 min. Inactivation of BCDH complex (ketoleucine) was correlated linearly with ^{32}P incorporation as shown in fig.2(b) (note inverted ordinate). Inactivation was apparently first order as shown in fig.2(c) and the rate constant was -0.904 ± 0.032 min^{-1} (mean ± SEM for 6 obs.; correlation coefficient, $r = 1.00$). Lastly, inactivation by ATP was reduced from 94% (in 2.5 min) to 20% by 2 mM ketoleucine.

4. Conclusions

The method of purification described has yielded BCDH complex which is rapidly phosphorylated and inactivated by ATP and which presumably contains a kinase associated with the complex. The overall yield of complex was poor (<6%) and much less than that given in [8] (44–100%). Success in obtaining BCDH complex inactivated and phosphorylated by ATP appears to be due to avoidance of MOPS buffer and to precipitation at pH 6.4 in the absence of TPP as opposed to precipitation at pH 6.2 in the presence of TPP. Further refinement may improve the yield but a suitable preparation for more detailed studies of phosphorylation/dephosphorylation and its regulation is now available.

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References

[1] Parker, P. J. and Randle, P. J. (1978) FEBS Lett. 95, 153–156.
[2] Odyssey, R. and Goldberg, A. L. (1979) Biochem. J. 178, 475–489.
[3] Parker, P. J. and Randle, P. J. (1980) FEBS Lett. 112, 186–190.
[4] Odyssey, R. (1980) Biochem. J. 192, 155–163.
[5] Lau, K. S., Fatania, H. R. and Randle, P. J. (1981) FEBS Lett. 126, 66–70.
[6] Randle, P. J., Lau, K. S. and Parker, P. J. (1981) in: Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids, (Walser and Williamson eds) pp. 13–22, Elsevier/North-Holland, Amsterdam, New York.
[7] Odyssey, R. (1980) FEBS Lett. 121, 306–308.
[8] Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1978) Proc. Natl. Acad. Sci. USA 75, 4881–4885.
[9] Hughes, W. A. and Halestrap, A. P. (1981) Biochem. J. 196, 459–469.
[10] Cooper, R. H., Randle, P. J. and Denton, R. M. (1974) Biochem. J. 143, 625–641.
[11] Sugden, P. H. and Randle, P. J. (1978) Biochem. J. 173, 659–668.
[12] Barrera, C. R., Namihara, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C. and Reed, L. J. (1972) Arch. Biochem. Biophys. 148, 343–358.