Activation of phosphorylated branched chain 2-oxoacid dehydrogenase complex

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Phosphorylation, activation of Differential regulation *Branched chain 2-oxoacid dehydrogenase Mitochondria, of rat liver, kidney, heart* *Activator, of BCDH complex BCDH complex, of ox kidney*

1. INTRODUCTION

The branched chain 2-oxoacid dehydrogenase complexes of ox kidney [1], rat heart, kidney and liver [2–6] and rabbit liver [7] are inactivated by phosphorylation with ATPMg and some aspects of the regulation of the kinase reaction have been described [8]. Little is known of reactivation of phosphorylated complex, although its occurrence in mitochondria is documented [2–4]. We show here that phosphorylated ox kidney BCDH complex is activated without dephosphorylation by a high speed supernatant fraction prepared from extracts of rat liver or kidney mitochondria. Activator was not detected in a similar fraction of rat heart mitochondria. Activator was thermolabile, non-diffusible by dialysis, inactivated by trypsin and precipitated by $(NH_4)_2SO_4$.

2. EXPERIMENTAL

Sources of chemicals, biochemicals, and [γ-^{32}P]-ATP were as in [1]. BCDH complex was purified partially from ox kidney mitochondria. The procedure was as in [1] incorporating the modification given in [8]. The final two steps in [1] (precipitations at pH 6.8 and 6.4) which complete separation of BCDH complex from pyruvate dehydrogenase complex were omitted, in order to increase the yield of BCDH complex (5–10-fold) and the rate of inactivation by MgATP (~4-fold). Apparent first order rate constants for inactivation by MgATP ranged from 1–3. Activity of pyruvate dehydrogenase complex was up to 80% of that of BCDH complex as estimated by the activity ratio with ketoleucine and pyruvate [1]. Pig heart pyruvate dehydrogenase complex was purified as in [9].

Mitochondria were prepared from rat heart as in [10], from rat liver and kidney as in [6] and from rat skeletal muscles with trypsin as in [11]. For preparation of HSS mitochondrial pellets were frozen (liquid N_2), thawed, and dispersed into extraction buffer (30 mM potassium phosphate/ 5 mM DTT/5 mM EGTA/1% (v/v) ox serum (pH 7.5); in some experiments 0.2 mM TPP). Protein (estimated as in [12] was adjusted to 50 mg/ml (exclusive of ox serum protein) by dilution with extraction buffer. After freezing and thawing ×2 extracts were centrifuged for 90 min at 150 000 × *g*. The supernatant was removed and, where necessary, stored at −10°C. Dialysis where performed, was against 100 vol. extraction buffer (without TPP) at 0°C for 24 h. For assay of BCDH complex and phosphorylated BCDH complex heart mitochondria were incubated for 10 min at 30°C in KCl medium [13] (2 mg mitochondrial protein/ ml), separated by centrifugation as in [13], frozen in liquid N_2 and extracts prepared as above. For preparation of extracts containing BCDH mitochondria were incubated without respiratory substrate. For preparation of extracts containing phosphorylated BCDH incubations were in 5 mM 2-oxoglutarate/0.5 mM L-malate.

Abbreviations: BCDH complex, branched chain 2-oxoacid dehydrogenase complex; DTT, dithiothreitol; EGTA, ethanedioxybis(ethylamine)tetraacetate; HSS, high-speed supernatant fraction; ketoleucine, 4-methyl 2-oxopentanoate; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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Phosphorylated ox kidney BCDH complex was prepared by incubation of BCDH complex (1 unit/ml) for 5 min at 30°C with 0.3–0.5 mM ATP in 30 mM potassium phosphate/5 mM DTT/5 mM EGTA/10 mM $MgCl_2$/1% (v/v) ox serum (pH 7.5). This resulted in ≥96% inactivation of BCDH complex by phosphorylation. Preparations were used either without further treatment (method A) or freed of adenine nucleotides and (where present) $^{32}P_i$ by dialysis followed by sedimentation of the complex through a 2% (w/v) sucrose layer (150 000 × *g*, 90 min) (method B); or by sedimentation without dialysis (method C). Dialysis resulted in some reactivation and dephosphorylation (up to 30%); sedimentation gave no reactivation of dephosphorylation. Phosphorylated pig heart pyruvate dehydrogenase complex was prepared by method A.

Rat heart BCDH complex was assayed spectrophotometrically by the initial rate of NADH formation with ketoleucine as substrate as in [6] (but with 30 units/ml lipoamide dehydrogenase). Ox kidney BCDH complex was assayed by the same method omitting Triton. Pyruvate dehydrogenase complex was assayed with 1 mM pyruvate in place of ketoleucine. In the presence of rat liver HSS (which contains lactate dehydrogenase) pyruvate dehydrogenase complex was assayed by coupling to arylamine acetyltransferase as in [12]. Assays of ^{32}P in phosphorylated complexes and of $^{32}P_i$, and SDS–PAGE and autoradiography were by methods given in [1,6,13].

One unit of enzyme activity forms 1 μmol substrate/min (NADH or acetylCoA) at 30°C. Values for K_a and V_m were computed as in [14]. (K_a is the concentration of HSS giving half-maximal reactivation of phosphorylated BCDH complex.) In what follows results are given as mean ± SEM; and HSS concentrations are given as mg mitochondrial protein from which they were made.

3. RESULTS AND DISCUSSION

3.1. *Specificity of assays*

Pyruvate dehydrogenase complex (present in BCDH complex used here) does not oxidize ketoleucine and does not interfere with the assay of BCDH complex. On incubation with ATP the 2 complexes were phosphorylated and inactivated concomitantly; with [γ-^{32}P]ATP in experiments to be described, incorporation of ^{32}P into the pyruvate dehydrogenase complex was ~30% of the total incorporation (SDS–PAGE and autoradiography). Under conditions of incubation used phosphorylated pyruvate dehydrogenase complex was neither reactivated nor dephosphorylated (not given).

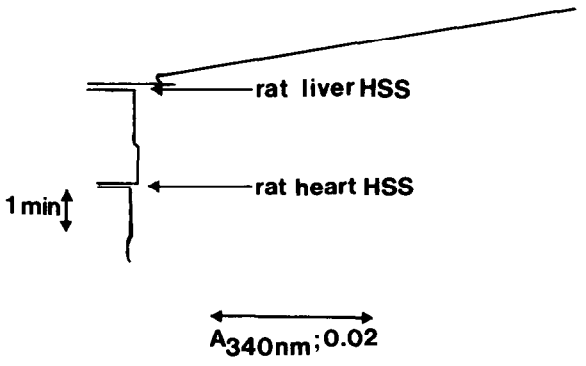

Fig.1. Effect of mitochondrial high-speed supernatant fraction (HSS) on the activity of phosphorylated ox kidney BCDH complex. Taken from the chart of a Gilford recording spectrophotometer omitting excursions due to absorbance of HSS and subsequent backing off. The cuvette contained in 1.4 ml, substrates, coenzymes and phosphorylated complex prepared by method C (equivalent to 20 munits BCDH complex). Rat heart HSS and dialysed rat liver HSS (50 μl equivalent to 2.5 mg mitochondrial protein) were added as shown. Neither HSS gave a measurable rate in the absence of phosphorylated complex (not shown).

3.2. *Activation of phosphorylated BCDH complex by mitochondrial HSS*

Addition of rat liver mitochondrial HSS to a cuvette, in which phosphorylated ox kidney BCDH complex showed no detectable activity, led within seconds to substantial reactivation of phosphorylated complex (fig.1). The rat liver HSS gave no measurable rate of NAD^+ reduction in the absence of phosphorylated complex. Reactivation has been observed consistently in >100 expt with 8 preparations of rat liver HSS and 12 preparations of phosphorylated complex made from 3 individual preparations of BCDH complex. Reactivation was independent of the order of addition, i.e., NAD^+ reduction could be initiated by the final addition of HSS (as in fig.1) or ketoleucine or phosphorylated complex (not shown), all other

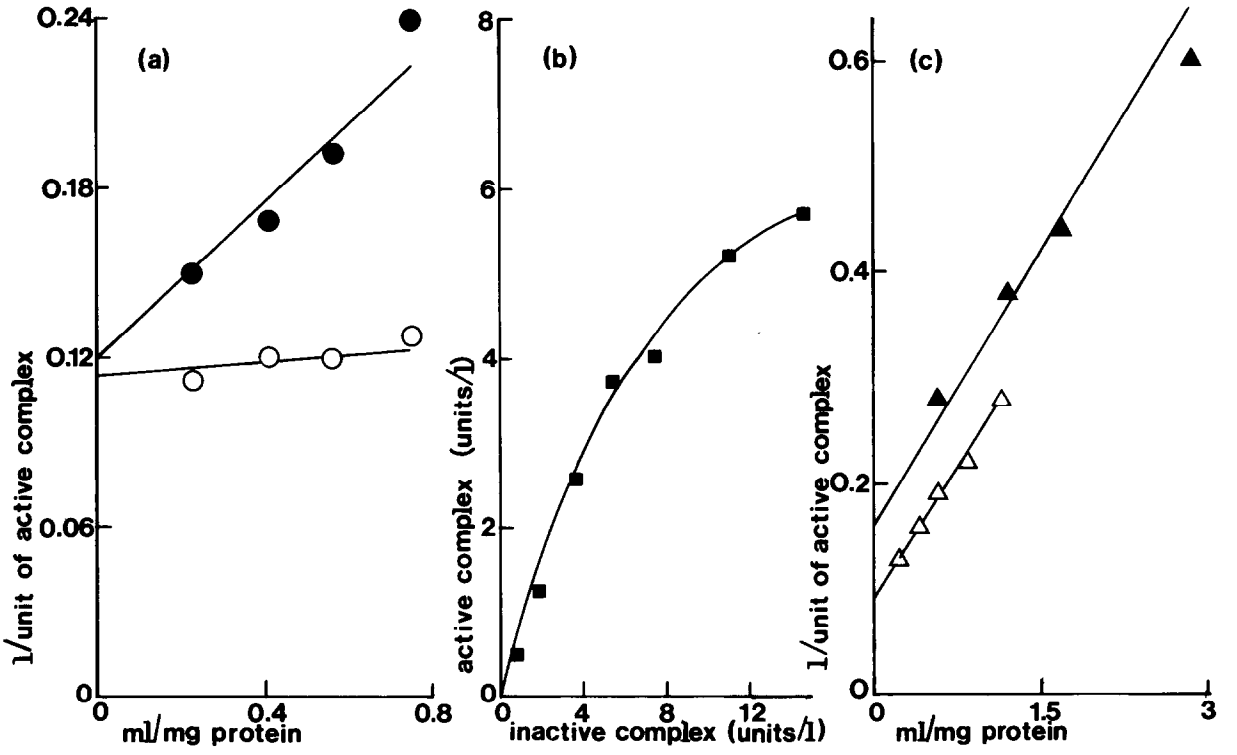

Fig.2. Effect of rat liver mitochondrial HSS on the activity of phosphorylated ox kidney BCDH complex prepared by method A. In all 3 panels concentrations of HSS (mg/ml) and of BCDH complex (units/l) are those in the cuvette. All points are means of 4 obs. (a) Double reciprocal plot showing effect of HSS concentration on activity of phosphorylated BCDH complex (•) or of BCDH complex (○). The activity of BCDH complex in the absence of HSS was 6.7 ± 0.11 units/l (reciprocal 0.15); that of phosphorylated BCDH complex was 0.03 ± 0.008 units/l (reciprocal 33). V_m values (units/l) were 8.51 ± 0.47 (•) and 8.94 ± 0.23 (○) ($P<0.001$ for effect of HSS in each case; $P>0.4$ for effect of phosphorylation in presence of HSS). The K_a values (mg mitochondrial protein/ml) were 1.23 ± 0.2 (•) and 0.17 ± 0.05 (○) ($P<0.001$ for difference). (b) Degree of activation of different concentrations of phosphorylated BCDH complex (inclusive of that present in the BCDH complex, as made) by a fixed concentration of rat liver HSS (1.77 mg/ml). (c) Double reciprocal plot as in (a) but with two different concentrations of phosphorylated complex (▲) 5 μl, and (△) 10 μl. V_m values were 6.2 ± 0.39 (5 μl) and 10.9 ± 0.28 (10 μl) ($P<0.001$ for difference); the K_a values were 1.08 ± 0.14 (5 μl) and 1.75 ± 0.10 (10 μl) ($P<0.001$ for difference).

components being present. Most preparations of rat liver HSS gave a blank rate in the absence of complex which required one or more of NAD^+, TPP and CoA but not ketoleucine. The blank rate was ~1.5 nmol · min^{-1} · 1.77 mg mitochondrial $protein^{-1}$ · ml^{-1}; i.e., 15% of the overall rate with 10 munits of fully reactivated phosphorylated complex. The technique adopted was to measure the blank rate first and to initiate the BCDH complex reaction by addition of phosphorylated complex. Reactivation of phosphorylated complex was independent of the method of preparation (A, B or C); A was the method of choice because the number of units of phosphorylated complex inactivated by phosphorylation is known by comparison with a control sample (no ATP) incubated in parallel.

Some preparations of rat liver HSS gave near complete reactivation when tested at 1.77 mg/ml (50 μl in 1.4 ml in cuvette). In a typical experiment (4 obs.) activities with phosphorylated complex equivalent to 9.3 ± 0.01 munits of BCDH complex were: 0.3 ± 0.01 munits (no HSS), 10.2 ± 0.05 munits (HSS, 1.77 mg/ml, corrected for blank rate). Some preparations of HSS gave incomplete reactivation at this concentration. Fig.2(a) shows the extent of reactivation of phosphorylated com-

plex and of BCDH complex (as isolated), by different concentrations of rat liver HSS (double reciprocal plot). The V_m values for BCDH complex as isolated, and for phosphorylated complex prepared from it did not differ significantly and were 30% greater than for unphosphorylated complex in the absence of HSS. This suggests that complex as isolated may contain ~20% of phosphorylated complex (values of V_m are in legend to fig.2(a).

The K_a values for HSS in fig.2(a) were 1.23 mg/ml (phosphorylated complex) and 0.17 mg/ml (complex as isolated). This suggested that the extent of reactivation may depend upon the concentration ratio [liver HSS]/[phosphorylated complex]. This suggestion is supported by data given in fig.2(b) in which the fractional reactivation declined as the ratio of [liver HSS]/[phosphorylated complex] was decreased. With 1.77 mg/ml HSS fractional reactivation was 63 ± 1.34% at 0.74 units/l and 39 ± 0.95% at 14.6 units/l phosphorylated complex (values inclusive of phosphorylated complex in BCDH complex as made). In fig.2(c) is shown the effect of increasing concentrations of liver HSS on the extent of reactivation of two concentrations of phosphorylated complex (5 μl and 10 μl in cuvette). The K_a value for 5 μl of phosphorylated complex was significantly less than that for 10 μl of phosphorylated complex.

Reactivation of phosphorylated ox kidney BCDH complex has been shown also with HSS from rat and ox kidney mitochondria (not given). No reactivation has been seen with HSS from rat heart mitochondria. Fig.1 shows the results of one such experiment. In a more detailed study (6 obs. each group) activities with phosphorylated complex equivalent to 7.2 ± 0.11 units/l were: phosphorylated complex (no HSS) 0.28 ± 0.02; phosphorylated complex (heart HSS, 1.77 mg/ml) 0.14 ± 0.04; phosphorylated complex (heart and liver HSS, each 1.77 mg/ml) 6.3 ± 0.07. This was repeated with comparable results (not given). In a single experiment no reactivation was seen with HSS from rat skeletal muscle mitochondria (1.77 mg/ml) (not shown).

In two separate experiments (total of 8 obs. in each group) rat liver HSS completely reactivated phosphorylated BCDH complex in extracts prepared from rat heart mitochondria incubated with 2-oxoglutarate/L-malate; no effect was seen on unphosphorylated complex in extracts prepared from mitochondria incubated without respiratory substrate (not shown).

Phosphorylated pig heart pyruvate dehydrogenase complex was not reactivated by rat liver HSS (not shown).

3.3. *Reactivation by rat liver HSS and protein bound [^{32}P]phosphate*

Phosphorylated ox kidney BCDH complex (method A; 7.4 ± 0.14 munits/ml in cuvette; prepared with [γ-^{32}P]ATP, 1100 dpm/pmol) was assayed for active complex (4 obs.) without HSS (0.35 ± 0.02 units/l) and with 4.43 mg/ml rat liver HSS (6.0 ± 0.21 units/l). Protein bound [^{32}P]phosphate was assayed on triplicate samples of 50 μl from each cuvette. The values (corrected for blanks, substituting an equivalent volume of [γ-^{32}P]ATP for phosphorylated complex) were (12 obs.) 721 ± 19 dpm (no HSS) and 733 ± 25 dpm (with HSS). This experiment shows that rat liver HSS does not release protein bound [^{32}P]phosphate as $^{32}P_i$, but does not prove that [^{32}P]phosphate remained in phosphorylated BCDH complex. In an attempt to show this 1 ml samples from the cuvette were centrifuged at 150 000 × *g* for 90 min to sediment phosphorylated complex and 50 μl of the supernatant assayed for protein bound [^{32}P]phosphate. The results (10 obs.) were 1230 ± 24 dpm (no HSS; before sedimentation); 1218 ± 15 dpm (1.77 mg/ml rat liver HSS; before sedimentation); 334 ± 38 dpm (no HSS; after sedimentation); 301 ± 27 dpm (HSS; after sedimentation). Apparently reactivation by HSS did not diminish protein bound ^{32}P which sedimented with the phosphorylated BCDH and pyruvate dehydrogenase complexes. In a further experiment with [^{32}P]phosphorylated complex prepared by method B trichloroacetic acid-soluble radioactivity was measured on 1 ml from the cuvette. In the absence of rat liver HSS activity was 3.23 ± 0.14 units/l and 4.8 ± 0.13% of ^{32}P was trichloroacetic acid-soluble; with rat liver HSS activity was 11.3 ± 0.13 units/l and 3.5 ± 0.07% of ^{32}P was trichloroacetic acid-soluble (total ^{32}P was 920 dpm/ml).

4. CONCLUSIONS

Rat liver, rat kidney and ox kidney mitochondria would appear to contain a factor which reacti-

vates phosphorylated ox kidney BCDH complex without dephosphorylation. This factor appears to be absent from rat heart and (one experiment) rat skeletal muscle mitochondria. The factor has yet to be characterised but it is known to be inactivated by heat (50°C for 10 min) and by trypsin; is non-diffusible by dialysis (24 h); and is precipitated by 0.7 saturation with $(NH_4)_2SO_4$ with a recovery of ~30% (not shown). This suggests that the factor may be a protein.

Three aspects of these findings are perhaps worthy of comment. The results of assays of BCDH complex in extracts of liver or kidney, or of mitochondria therefrom, may require re-evaluation because of the possibility of varying degrees of activation by the factor, depending on the experimental conditions. This stricture may not apply to assays in extracts of heart muscle or heart muscle mitochondria. When purified, activator may afford a useful means of determining the proportions of active and inactive complex in tissues. The tissue distribution of activator may lend support to the idea of differential regulation of the activity of BCDH complex in liver and kidney as opposed to muscles [2,3,6,15,16]. Physiological significance of activator may be suggested by the finding that it is fully effective at a ratio of [phosphorylated complex]/[liver HSS] of 5.25 munits/mg mitochondrial protein. The measured activity of BCDH complex (active form) in rat liver mitochondria was 6 munits/mg protein in [6]. Other interesting questions, such as whether activator is an inhibitor of the BCDH kinase reaction [6] may be answered after purification and characterisation.

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