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Purification and Crystallization of the Recombinant Catalytic Subunit of Pyruvate Dehydrogenase Phosphatase

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Pyruvate Dehydrogenase Phosphatase의 Catalytic Subunit의 분리정제 및 결정화

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ABSTRACT – Pyruvate dehydrogenase phosphatase (PDP) is a mitochondrial protein serine/threonine phosphatase that catalyzes the dephosphorylation and concomitant reactivation of the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex (PDC). PDP consists of a catalytic subunit (PDPc, Mr 52,600) and regulatory subunit (PDPr, Mr 95,600). In the presence of Ca^{2+}, PDPc binds to the dihydrolipoamide acetyltransferase (E2) component of the pyruvate dehydrogenase complex in proximity to its substrate, the phosphorylated E1 component, thereby increasing the rate of dephosphorylation. PDPc possesses an intrinsic Ca^{2+} binding site and a second Ca^{2+} site is generated in the presence of E2. Using the unique interaction, highly pure PDPc was produced by the GSH-Sepharose-GST-L2 matrix with a specific activity of approx. 1000 U/mg and a yield of about 80%.

Key words : pyruvate dehydrogenase phosphatase, catalytic subunit of pyruvate dehydrogenase phosphatase, crystallization, purification

Pyruvate dehydrogenase phosphatase (PDP) is a mitochondrial protein serine/threonine phosphatase that catalyzes the dephosphorylation and concomitant reactivation of the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex (PDC) (1, 2). PDP consists of a Mg^{2+}-dependent and Ca^{2+}-stimulated catalytic subunit (PDPc) of *M*r 52,600 and an FAD-containing regulatory subunit (PDPr) of *M*r 95,600 (2-5). PDPc is a member of the protein phosphatase 2C family (6). Ca^{2+} mediates the translocation of PDP (or PDPc) to the 60-mer icosahedral dihydrolipoamide acetyltransferase (E2) component of the PDC in proximity to its substrate, the phosphorylated pyruvate dehydrogenase component (E1), thereby increasing the rate of dephosphorylation about 10-fold (2, 7, 8). The site od Ca^{2+}-facilitated binding of PDP and PDPc is the inner lipoyl domain (L2) of E2 (8).

The rPDPc was purified using several steps of SP-sepharose, dialysis, DEAE-sepharose, and Superose HR12 (6). Through the protocol, rPDPc was purified with a low yield (0.8 3.3 mg of rPDPc per 10 g of cell) and a low specific activity (around 300 nmole/min/mg protein). In 1996, Yan *et al.* reported that native PDP was bound to E2 of PDC in the presence of Ca^{2+} (5). The report showed that both PDP and PDPc bound to E2 in the presence, but not in the absence of Ca^{2+}. And also, Chen *et al.* reported that native PDP bound to inner lipoyl domain (L2) of E2 of PDC in the presence of Ca^{2+}(8).

In this paper, we report that rPDPc is purified to apparent homogeneity using the unique interaction between rPDPc and L2 and crystals that diffract to 2.7 Å have been obtained with current preparation of PDPc.

Materials and Methods

Materials

Escherichia coli strain BL21(DE3) harboring a plasmid encoding L2 (amino acids 120-233 of E2) fused to

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GST was kindly provided by Drs Thomas E. Roche and Jason C. Baker (Kansas State University). To make the affinity matrix for purification, GSH-Sepharose 4B was obtained from Pharmacia and [γ-^{32}P]ATP was from New England Nuclear.

Expression of the Catalytic Subunit of Pyruvate Dehydrogenase Phosphatase (rPDPc)

The PDPc construct was transformed with p1922 plus pGroESL (pGroESL encodes *E. coli* chaperonin proteins groEL and groES). Fresh transformants were grown at 30°C to an OD_{600} of 0.6~1.8. Expression was induced by addition of IPTG to a final concentration of 0.5 mM. Incubation was continued at 30°C for 5.5~6.5 h. Cells were spun down and used for the purification of PDPc.

Expression of the GST-Lipoyl domain of E2 of PDC and preparation of affinity matrix (GSH-Sepharose GST-L2)

GST-L2 fusion protein was expressed in *E. coli* BL21(DE3) and purified essentially as described (4, 6). Briefly, the fragment was subcloned into pGEX-2T and then this plasmid was transformed into *E. coli* strain $BL_{21}(DE_3)$. The *E. coli* BL21(DE3) strain transformed with the pGEX-2T containing L2 (amino acids 120-233 of E2) construct was grown in 0.5% Yeast extract, 1% trypton, 1% NaCl, 0.1% glucose, 5 mM Na_2HPO_4, 100 μg/ml ampicillin, and 0.2 mM DL-α-lipoate. The culture was incubated at 37°C to an OD_{600} of 0.8~1.0. Expression was induced with IPTG to a final concentration of 0.5 mM, and growth was continued at 37°C for 5.5~6.5 h. Cells for L2-GST were suspended in 9 volume of ice-cold PBST buffer (20 mM sodium phosphate, pH 7.2, 0.15 M NaCl, 1.0% triton X-100) and disrupted by two passes with French press cell disrupter at a pressure of around 800 psi, and then it was centrifuged at 48,400 × g for 30 min. The extract was mixed with the GSH-Sepharose 4B that had been equilibrated with PBST buffer. The mixture was mildly shaken for 30 min at room temperature, and then it was loaded into an empty column. The column was washed with 10-column volume of PBST and then equilibrated with ice-cold buffer K (50 mM tris HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.15 M NaCl, 1 mM benzamidin).

Purification of rPDPc

PDPc was expressed in *E. coli* by the procedure described (11), with minor modification. Briefly, cells were suspended in 9 volume of ice-cold buffer K (50 mM tris HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.15 M NaCl, and 1 mM benzamidin) and disrupted by one pass with French press cell disrupter at a pressure of around 1000 psi, and then it was centrifuged at 48,400 × g for 30 min. The extract was loaded on L2-GST*GSH-Sepharose 4B equilibrated with buffer K containing 1% triton X-100 and 1 M urea. It was washed extensively with buffer K containing 1% triton X-100 and 1 M urea and followed to wash with buffer K without 10% glycerol, and 0.15 M NaCl. And rPDPc was eluted with elution buffer (50 mM tris HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM benzamidin, and 1 mM EGTA).

Assay of rPDPc

Measurements of rPDPc activity were based on the measurement of the initial rate of release of [^{32}P]phosphate from the bovine ^{32}P-labeled pyruvate dehydrogenase complex (9). One unit is defined as the amount of phosphate that releases 1 nmole [^{32}P]phosphate/min.

SDS-PAGE and Immunoblotting

Proteins were separated by SDS-PAGE on 12.5% gels and transferred electrophoretically to Immobilon-P membrane (Millipore). Blots were probed with a 1:2000 dilution of rabbit anti-rPDPc IgG followed by detection with 1:2000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase as described by the supplier (Bio-Rad)

Crystallization of purified PDPc

Crystals of rPDPc were obtained from protein exchanged into 50 mM HEPES buffer (pH 7.3) as follows. Conditions for crystallization were screened by the spare matrix technique with the hanging drop method (12) using the Hampton's Crystal Screen II Kit (Table 1).

Other methods

Protein was determined based on Bradford methods (10), using bovine serum albumin as the standard.

Table 1. Crystal Screen Reagent Formulations

1	30% MPD, 0.1 M Sod. acetate pH 4.6, 0.02 M Calcium chloride
2	0.4 M Sod. Tartrate
3	0.4 M Ammonium phosphate
4	2.0 M Ammonium sulfate, 0.1 M Tris HCl pH 8.5
5	30% MPD, 0.1 M Na HEPES pH 7.5, 0.2 M Sod. Citrate
6	30% PEG 4000, 0.1 M Tris HCl pH 8.5, 0.2 M Mg chloride
7	1.4 M Sod. acetate, 0.1 M Sod. cacodylate pH 6.5
8	30% 2-Propanol, 0.1 M Sod. cacodylate pH 6.5, 0.2 M Sod. citrate
9	30% PEG 4000, 0.1 M Sod. citrate pH 5.6, 0.2 M Ammonium acetate
10	30% PEG 4000, 0.1 M Sod. acetate pH 4.6, 0.2 M Ammonium acetate
11	1.0 M Ammonium phosphate, 0.1 M Sod. citrate pH 5.6
12	30% 2-Propanol, 0.1 M Sod. HEPES pH 7.5, 0.2 M Mg chloride
13	30% PEG 400, 0.1 M Tris HCl pH 8.5, 0.2 M Sod. citrate
14	28% PEG 400, 0.1 M Sod. HEPES pH 7.5, 0.2 M Calcium chloride
15	30% PEG 8000, 0.1 M Sod. Cacodylate pH 6.5, 0.2 M Ammonium sulfate
16	1.5 M Li sulfate, 0.1 M Sod. HEPES pH 7.5
17	30% PEG 4000, 0.1 M Tris HCl pH 8.5, 0.2 M Li sulfate
18	20% PEG 8000, 0.1 M Sod. Cacodylate pH 6.5, 0.2 M Mg acetate
19	30% 2-propanol, 0.1 M Tris HCl pH 8.5, 0.2 M Ammonium acetate
20	25% PEG 4000, 0.1 M Sod. Acetate pH 4.6, 0.2 M Ammonium sulfate
21	30% MPD, 0.1 M Sod. Cacodylate pH 6.5, 0.2 M Mg acetate
22	30% PEG 4000, 0.1 M Tris HCl pH 8.5, 0.2 M Sod. Acetate
23	30% PEG 400, 0.1 M Sod. HEPES pH 7.5, 0.2 M Mg chloride
24	20% 2-propanol, 0.1 M Sod. Acetate pH 4.6, 0.2 M Calcium chloride
25	1.0 M Sod. Acetate, 0.1 M Imidazole pH 6.5
26	30% MPD, 0.1 M Sod. Citrate pH 5.6, 0.2 M Ammonium acetate
27	20% 2-Propanol, 0.1 M Sod. HEPES pH 7.5, 0.2 M Sod. Citrate
28	30% PEG 8000, 0.1 M Sod. Cacodylate pH 6.5, 0.2 M Sod. Acetate
29	0.8 M K, Na Tartrate, 0.1 M Sod. HEPES pH 7.5
30	30% PEG 8000, 0.2 M Ammonium sulfate
31	30% PEG 4000, 0.2 M Ammonium sulfate
32	2.0 M Ammonium sulfate
33	4.0 M Sod. Formate
34	2.0 M Sod. Formate, 0.1 M Sod. Acetate pH 4.6
35	1.6 M Sod. Phosphate, 0.1 M od. HEPES pH 7.5
36	8% PEG 8000, 0.1 M Tris HCl pH 8.5
37	8% PEG 4000, 0.1 M Sod. Acetate pH 4.6
38	1.4 M Sod. Citrate, 0.1 M Sod. HEPES pH 7.5
39	2% PEG 400, 2.0 M ammonium sulfate, 0.1 M Sod. HEPES pH 7.5
40	20% 2-propanol, 20% PEG 4000, 0.1 M Sod. Citrate pH 5.6
41	10% 2-Propanol, 20% PEG 4000, 0.1 M Sod. HEPES pH 7.5
42	20% PEG 8000, 0.05 M K Phosphate
43	30% PEG 1500
44	0.2 M Mg formate
45	18% PEG 8000, 0.1 M Sod. Cacodylate pH 6.5, 0.2 M Zn acetate
46	18% PEG 8000, 0.1 M Sod. Cacodylate pH 6.5, 0.2 M Ca acetate
47	2.0 M Ammonium sulfate, 0.1 M Sod. Acetate pH 4.6
48	2.0 M Ammonium phosphate, 0.1 M Tris HCl pH 8.5
49	2% PEG 8000, 1.0 M Li sulfate
50	15% PEG 8000, 0.5 M Li sulfate
51	10% PEG 6000, 2.0 M Sod. Chloride
52	0.5 M NaCl, 0.01 M CTAB, 0.01 M Mg chloride
53	25% ethylene glycol
54	35% Dioxane
55	5% Isopropanol, 2.0 M Ammonium sulfate

Table 1. Continued

56	1.0 M Imidazole pH 7.0
57	10% PEG 1000, 10% PEG 8000
58	10% Ethanol, 1.5 M Sod. Chloride
59	2.0 M Sod. Chloride, 0.1 M Sod. Acetate pH 4.6
60	30% MPD, 0.1 M Sod. Acetate pH 4.6, 0.2 M NaCl
61	1.0 M 1,6-Heanediol, 0.1 M Sod. Acetate pH 4.6, 0.01 M Co chloride
62	30% PEG 400, 0.1 M Sod. Acetate pH 4.6, 0.1 M Cd chloride
63	30% PEG MME 2000, 0.1 M Sod. Acetate pH 4.6, 0.2 M Ammonium sulfate
64	2.0 M Ammonium sulfate pH 5.6, 0.2 M K/Na tartrate
65	1.0 M Li sulfate, 0.1 M Na citrate pH 5.6, 0.5 M Ammonium sulfate
66	2% Polyethyleneimine, 0.1 M Sod. Citrate pH 5.6, 0.5 M Sod. Chloride
67	35% tert-butnol, 0.1 M Sod. Citrate pH 5.6
68	10% Jeffamine M-600, 0.1 M Sod. citrate pH 5.6, 0.0 M Ferric chloride
69	2.5 M 1,6-Hexanediol, 0.1 M Sod. citrate pH 5.6
70	1.6 M Mg sulfate, 0.1 M MES pH 6.5
71	2.0 M Sod. chloride, 0.1 M MES pH 6.5, 0.2 M Na/K phosphate
72	12% PEG 20,000, 0.1 M MES pH 6.5
73	10% Dioxane, 0.1 M MES pH 6.5, 1.6 M Ammonium sulfate
74	30% Jeffanine M-600, 0.1 M MES pH 6.5, 0.p5 M Cs chloride
75	1.8 M Ammonium sulfate, 0.1 m MES pH 6.5, 0.01 M Co chloride
76	305 PEG MME 5000, 0.1 M MES pH 6.5, 0.2 M Ammonium sulfate
77	25% PEG MME 550, 0.1 M MES pH 6.5, 0.01 M Zn sulfate
78	1.6 M Sod. citrate pH 6.5
79	30% MPD, 0.1 M HEPES pH 7.5, 0.5 M Ammonium sulfate
80	10% PEG 6000, 0.1 M HEPES pH 7.5, 5% MPD
81	20% Jeffamine M-600, 0.1 M HEPES pH 7.5
82	1.6 M Ammonium sulfate, 0.1 M HEPES pH 7.5, 0.1 M Na chloride
83	2.0 M Ammonium formate, 0.1 M HEPES pH 7.5
84	1.0 M Sod. acetate, 0.1 M HEPES pH 7.5, 0.05 M Cd sulfate
85	70% MPD, 0.1 M HEPES pH 7.5
86	4.3 M Sod. chloride, 0.1 M HEPES pH 7.5
87	10% PEG 8000, 0.1 M HEPES pH 7.5, 8% Ethylene glycol
88	20% PEG 10,000, 0.1 M HEPES pH 7.5
89	3.4 M 1,6-Hexanediol, 0.1 M Tris pH 8.5, 0.2 m Mg chloride
90	25% tert-butanol, 0.1 M Tris pH 8.5, 0.1 M Ca chloride
91	1.0 M Li sulfate, 0.1 M Tris pH 8.5, 0.01 M Ni chloride
92	12% glycerol, 0.1 M Tris pH 8.5, 1.5 M Ammonium sulfate
93	50% MPD, 0.1 M Tris pH 8.0, 0.2 M Ammonium phosphate
94	20% Ethanol, 0.1 M Tris pH 8.5
95	20% PEG MME 2000, 0.1 M Tris pH 8.5, 0.01 M Ni chloride
96	30% PEG MME 550, 0.1 M Bicine pH 9.0, 0.1 M Sod. chloride
97	2.0 M Mg chloride, 0.1 M Bicine pH 9.0
98	10% PEG 20,000, 0.1 M Bicine pH 9.0, 2% Diocxane

Results and Discussion

The reaction catalyzed by the mammalian pyruvate dehydrogenase complex (PDC) links glycolysis with several biochemical pathways. In tissues with large energy demands like brain, muscle, and heart, it supplies the carbon units derived from carbohydrate fuels mainly for complete oxidation by the Krebs cycle. In lipogenic tissues such as adipose, mammary gland, and liver, the metabolic fate of acetyl-CoA derived from carbohydrates is quite different, because it can be used fro the biosynthesis of fatty acids and cholesterol (for reviews, see 13, 14). Therefore, the reaction catalyzed by PDC should be highly regulated, and this regulation must be sophisticated enough to acceommodate the different metabolic requirements of a variety of tissues. A most important mechanism of PDC regulation is based on reversible phosphorylation and dephosphorylation by

pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP), respectively (1). However, the structural characteristics of PDP has not been described, yet. Meanwhile, Choi, *et al.* (11) reported the way to purify catalytic subunit of PDP ustilizing GST-L2 affinity matrix. Utilizing the affinity gel, we reproducilbly purified almost homogenous PDPc like that purified by Choi, *et al.* and screened the condition for obtaining good crystal. The analysis of crystal is underway to determine the structure.

Purification of PDPc

Cloning and expression in *E. coli* of cDNA encoding bovine PDPc provided larger amount of this phosphatase. The finding that PDP binds in the presence of Ca^{2+} to the inner lipoyl domain (L2) of mammalian E2 led to development of a more efficient affinity chromatographic matrix for purification of PDP, namely a GST-L2 fusion protein bound to GSH-Sepharose 4B (Fig. 1). Utilizing the matrix, PDPc was purified to near homogeneity by the protocol (Fig. 2). When analyzed by SDS-PAGE and immunoblotting, highly purified preparation of recombinant PDPc showed a major band with Mr ~50,000 and variable amounts of a minor band with Mr ~45,000. The amino-terminal sequences of the two proteins were identical (data not shown). rPDPc was purified with a specific activity of ~1000 U/mg and a yield of about 80%. Approximately 40 mg of pure PDPc was obtained from 20 g (wet weight) of transformed *E. coli* cells. About two-thirds of the expressed PDPc was soluble, and the yield of highly purified PDPc was about 10 times that obtained by the previous multistep protocol. While highly purified PDPc obtained by this procedure contained about 5% nicked PDPc, PDPc produced

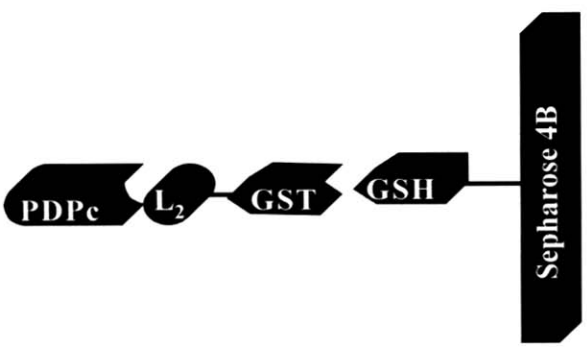

Fig. 1. Affinity resin for the purification of rPDPc.

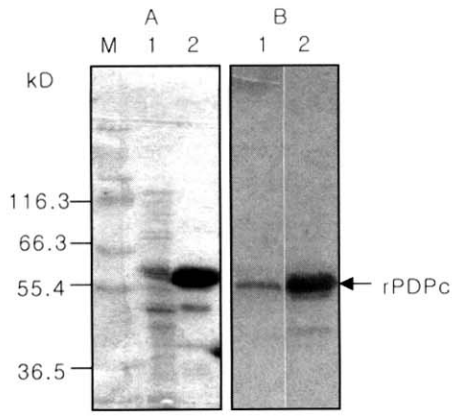

Fig. 2. SDS-PAGE and Immunoblot analysis of purified rPDPc. Purified rPDPc (6.7 g) was electrophored on 12.5% SDS- or Native-polyacrylamide gel and was stained with coomasie brilliant blue G. A: SDS-PAGE, B: Immunoblot analysis (lane M, molecular weight marker; lane 1, soluble cell extract; lane 2, purified rPDPc)

by the multistep protocol contained nicked PDPc more than 15% (6). Crystals of PDPc was obtained with the present preparation after ultrafiltration to concentrate to 20~30 mg/ml.

Crystallization of rPDPc

The conditions for crystallization were refined, and high-resolution, data-quality crystals were subsequently grown at 4°C using the sitting-drop method, where 15 uL of the protein [20~30 mg/mL rPDPc in 50 mM HEPES buffer (pH 7.3)] was mixed with 5 uL of 20% Jeffamin

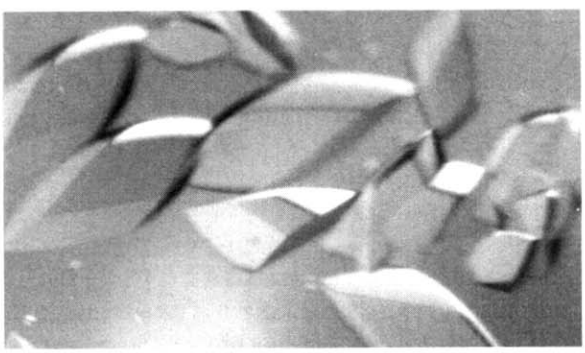

Fig. 3. Photograph of the crystal of purified rPDPc. The conditions for crystallization were refined, and high-resolution, data-quality crystals were subsequently grown at 4°C using the sitting-drop method, where 15 uL of the protein [20~30 mg/mL rPDPc in 50 mM HEPES buffer (pH 7.3)] was mixed with 5 uL of 20% Jeffamin M-600 as the precipitant.

M-600 as the precipitant. Crystals grew to a mature size of 0.4 mm 0.4 mm 0.5 mm within 4 weeks. As shown in Fig. 3, the crystal was hexagonal with cell constants a=b=75.3 Å and c=172 Å. The crystals diffract with 2.7 Å resolution. Most of the crystals tested are sensitive to X-ray, exhibiting a life-time of 35 minutes after x-irradiation. Therefore, long-lasting and higher quality crystals are necessary to obtain enough data to analyze the structural data and we are trying to modify crystal growth conditions.

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국문요약

당 대사에 관여하는 Pyruvate dehydrogenase phosphatase(PDP)는 해당과정에서의 대사 산물인 pyruvate 를 acetyl CoA로 만들어 구연산 회로로 진입시켜주는 효소인 pyruvate dehydrogenase complex(PDC)의 활성을 조절하는 중요한 효소이다. PDP의 catalytic subunit는 PDC의 dihydrolipoamide acetyltransferase(E2), PDP regulatory subunit (PDPr), 그리고 칼슘 결합 도메인 등으로 구성되어 있는 것으로 추측되어지고 있다. 본 연구에서는 PDP 단백질을 분리정제하고 결정화 하고자하였다. PDP는 catalytic subunit(PDPc, Mr 52,600 Da)과, regulatory subunit (PDPr, 95,600 Da)으로 구성되어 있으며 칼슘 존재하에 PDPc는 dihydrolipoamide acetyltransferase(E2) component와 결합하여 기질인 인산 E1 component의 탈인산화율을 증가시킨다. PDPc는 intrinsic 칼슘 결합부위를 가지며 두 번째 칼슘 부위는 E2 존재 하에 형성된다. 이러한 특이한 상호반응을 이용한 GSH-Sepharose-GST-L2 matrix를 이용하여 약 1000 U/mg의 specific activity를 갖는 순수 PDPc를 약 80%의 yield로 얻어 결정화에 사용하였다.

References

1. Linn, T.C., Pettit, F.H. and Reed, L.J.: -Keto acid Dehydrogenase Complex, X. Regulation of the activity of the Pyruvate Dehydrogease Complex from beef kidney mitochondria by phosphorylation and dephosphorylation. *Proc. Natl. Acad. Sci.*, **62**, 234-241 (1969).
2. Teague, W.M., Pettit, F.H., Wu, T.-L., Silberman, S.R. and Reed, L.J.: Purification and Properties of Pyruvate Dehydrogenase Phosphatase from Bovine Heart and Kidney. *Biochemistry*, **21**, 5585-5592 (1982).
3. Pratt, M.L., Maher, J.F. and Roche, T.E.: Purification of Bovine Kidney and Heart Pyruvate Dehydrogenase Phosphatase on Sepharose derivatized with Pyruvate Dehydrogenase Complex. *Eur. J. Biochem.*, **125**, 349-355 (1982).
4. Denton, R.M., Randle, P.J. and Martin, B.R.: Simulation by calcium ion of Pyruvate dehydrogenase phophate phosphatase. *Biochem. J.*, **128**, 161-163 (1972).
5. Yan, J., Lawson, J.E. and Reed, L.J.: Role of the regulatory subunit of bovine pyruvate dehydrogenase phosphatase. *Proc. Natl. Acad. Sci.*, **93**, 4953-4956 (1996).
6. Lawson, J.E., Niu, X.-D., Browning, K.S., LeTrong, H., Yan, J. and Reed, L.J.: Molecular cloning and Expression of the Catalytic Subunit of Bovine Pyruvate Dehydrogenase Phosphatase and Sequence Similarity with Protein Phosphatase 2C. *Biochemistry*, **32**, 8987-8993 (1993).
7. Pettit, F.H., Roche, T.E. and Reed, L.J.: Function of calcium ions in pyruvate dehydrogenase phosphatase activity. *Biochem. Biophy. Res. Comun.*, **49**, 563-571 (1972).
8. Chen, G., Wang, L., Liu, S., Chuang, C. and Roche, T.E.: Activated Function of the Pyruvate Dehydrogenase Phosphatase through Ca^{2+}-facilitated Binding to the Inner Lipoyl Domain of the Dihydrolipoyl Acetyltransferase. *J. Biol. Chem.*, **271**, 28064-28070 (1996).
9. Damuni, Z., Humphreys, J.S. and Reed, L.J.: Stimulation of pyruvate dehydrogenase phosphatase activity by polyamines. *Biochem. Biophy. Res. Comun.*, **124**, 95-99 (1984).
10. Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254 (1976).

11. Choi, W.S., Yan, J., McCarthy, D.B., Park, S.H. and Reed, L.J.: One step purification of the recombinant catalytic subunit of Pyruvate dehydrogenase phosphatase. *Protein Expression and Purification*, **20**, 128-131 (2000).
12. McPherson, A.: Current approaches to macromolecular crystallization. *Eur. J. Biochem.*, **189**, 1-23 (1990).
13. Rand;e, P. J.: Metabolic fuel selection: general integration at the wholebody level. *Proc. Nutr. Soc.*, **54**, 317-327 (1995).
14. Sugden, M. C., Orfali, K. A. and Holness, M. J.: The pyruvate dehydrogenase complex: nutrient control and the pathogenesis of insulin resistance. *J. Nutr.*, **125**, (suppl.) 1746S-1752S (1995).