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Supplemental Material to

INTERDOMAIN INFORMATION TRANSFER DURING SUBSTRATE ACTIVATION OF YEAST PYRUVATE DECARBOXYLASE: THE INTERACTION BETWEEN CYSTEINE 221 AND HISTIDINE 92.

by

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Experimental Procedures.

Construction of H92A, H92G, H92K, H92C/C222S, C221A/C222A, C222A variants of PDC: the procedure followed the instructions for Chameleon™ Double-Stranded Site-Directed Mutagenesis Kit from Stratagene (Papworth et al., 1995, Baburina et al., 1996).

The pSIT-*PDC1* vector possesses four *Afl*III restriction sites and produces 0.6 kb, 0.3 kb, 4 kb and 2.9 kb fragments upon digestion. As a result of site-directed mutagenesis, one *Afl*III restriction site is eliminated and the resulting fragments are 0.6 kb, 4 kb and 3.2 kb.

Substitutions in position 92 were confirmed by sequence analysis using primer 5'-CCGCGAAATTAATACGACTCACTATAGGG-3'.

Mutations in positions 221 and 222 were identified by sequencing, using primer 5'-CTGCTATGATCACTGACATCTGTACGCC-3'. Conversion of H92 to C was carried out on the *PDC1* gene already carrying the C222S substitution to yield the H92C/C222S doubly substituted variant. The screening was conducted using *Afl*III restriction digest according to the protocol described above.

The following primers were used for site-directed mutagenesis:

Substitution	Oligonucleotide Used	Codon Change
H92A	5'-CTTACGCTGAAGCCGTCGGTGTTTTGC-3'	CAC to GCC
H92G	5'-CTTACGCTGAAGGCGTCGGTGTTTTGC-3'	CAC to GGC
H92K	5'-CTTACGCTGAAAAGGTCGGTGTTTTGC-3'	CAC to AAG
H92C	5'-CTTACGCTGAATGCGTCGGTGTTTTGC-3'	CAC to TGC
C222A	5'-TGATGCTTGTGCTTCCAGACACGAC-3'	TGT to GCT
C221A/ C222A	5'-TGATGCTGCTGCTTCCAGACACGAC-3'	TGTTGT to GCTGCT

PDC overexpression and purification: WT PDC and its variants subcloned into pET1120 or pSIT vectors were transformed into *E. coli* BL21(DE3) strain for expression (Studier & Moffatt, 1986). The procedure for the transformation is standard and is described above in the section describing site-directed mutagenesis. *E. coli* cells were grown on LB medium containing 1 mM KPi buffer, pH 7.0, 2 mM ThDP and 2 mM Mg^{2+}. Cell culture was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for pET vectors and 1 mM IPTG for pSIT vectors for 4-6 h at late log phase (A_{600} >2.5).

Due to a higher amount of activity present in the crude extracts of PDC expressed in *E. coli* compared to PDC activity in crude extracts of yeast, the purification procedure was slightly modified from that of Farrenkopf & Jordan (1992).

After induction, 4 - 5 g of cells were resuspended in 50 mL of 20 mM KPi, pH 6.8 containing 2 mM EDTA and 4 mM $MgSO_4$ (buffer A). The following components were added to the buffer immediately prior to use: 5 mM β-mercaptoethanol, 5 mM phenylmethanesulfonyl fluoride (PMSF), 0.05% (v/v) Triton X-100 and 10 mM ThDP (stock solution of 100 mM ThDP can be prepared in 0.1M KPi buffer, pH 6.8; it can be kept in the refrigerator for no more than a week). Cell suspension was sonicated for 5 min on a 550 Sonic Dismembrator from Fisher Scientific at 20 KHz and amplitude setting 6. Cell debris was removed by centrifugation at 20,000 rpm for 25

min at 4 °C on a Beckman J2-21 centrifuge and the supernatant was saved. Streptomycin sulfate was added to the supernatant to a final concentration of 2% (w/v) out of a 50% (w/v) stock solution to remove DNA that would interfere with protein binding to the DEAE column during later stages of the purification procedure. The pellet was removed by centrifugation at 20,000 rpm for 5 min at 4 °C on the Beckman J2-21 centrifuge immediately upon addition of streptomycin and the supernatant was saved. 2.5 M Ammonium sulfate was slowly added to the supernatant; the suspension was allowed to stir for 15 - 20 min at room temperature and the pellet was collected at 20,000 rpm for 15 min at 20 °C on the Beckman J2-21 centrifuge. The pellet was resuspended in 5-7 mL of buffer A without Triton X-100 and dialyzed against the same buffer overnight. After dialysis, the solution was centrifuged at 20,000 rpm for 5 min at 4 °C on the Beckman J2-21 centrifuge. The resulting clear yellowish solution contains approximately 300 mg of protein with a PDC specific activity no less than 20 U/mg in a volume of 7-12 mL. The solution was loaded onto a conventional column packed with DEAE Sepharose Fast-Flow (80 mL bed volume). The column with the sample bound was first washed with buffer A without Triton X-100 and ThDP at a flow rate of 4 mL/min to remove non-specifically bound proteins. A linear gradient with 200 mM KPi, containing 2 mM EDTA, 4 mM $MgSO_4$ and 5 mM PMSF (buffer B) was applied at the same rate for 1 hour to a 100% of the second buffer. The gradient was generated by the Maxima program from Waters ™ with HPLC pumps from Waters™.

Samples were collected into tubes containing 100 mM ThDP in 0.1 M KPi buffer, pH 6.8 (pH of the ThDP solution should be adjusted to 6.8). The fractions were assayed for protein content and PDC activity and checked for purity using SDS-PAGE electrophoresis; fractions containing PDC with specific activity no less than 40 U/mg were combined and transferred into 100 mM KPi, pH 6.3 containing 10 mM ThDP, 10 mM Mg^{2+}, 5 mM PMSF and 0.1% (v/v) Triton X-100. 20% glycerol was added to the protein samples for long-term storage if necessary. Under these conditions, PDC remains stable for at least 6 months at 4 °C, or for more than a year at -20 °C.

A heat-treatment step (Kuo et al., 1986; Farrenkopf & Jordan, 1992) can be introduced into this protocol. However, some PDC variants did not survive the heat treatment step, therefore, this step was not used in these series of experiments in order to assure the same quality of all samples.

Optimization of expression of scpdc1 in E. coli. The overexpression was optimized for WT PDC and appeared to be satisfactory for all variants. Small aliquots of BL21 *E. coli* cells with the pET1120-*PDC1* plasmid (the gene for *Saccharomyces cerevisiae* PDC, scpdc1) were grown on LB medium and induced with IPTG at different values of A_{600}. After 4 hours of induction with IPTG, cells were pelleted, resuspended in a small amount of buffer A, sonicated and evaluated for PDC activity (data not shown). The highest amount of PDC activity is obtained from the cell culture that has reached late log phase of A_{600}>2.5. Further increase of the induction time did not lead to a significant increase in PDC activity, therefore all expression procedures were carried out with 4-6 hours of IPTG induction.

Optimization of the purification procedure. Typical purification of PDC from 1 liter of *E. coli* culture (4-5 g of cells) yields 8,000-10,000 units of PDC activity with specific activity of at least 10 U/mg, significantly higher than in the crude extract from yeast (3 U/mg, Farrenkopf & Jordan, 1992). With such an improved specific activity, some of the steps in the purification procedure developed for the yeast enzyme could be omitted. Table S1 compares the purification procedure for PDC overexpressed in *E. coli* (from 1 liter of cell culture) with the protocol for PDC from 50 g of yeast paste.

Figure S1 shows a typical elution profile of WT PDC overexpressed in *E. coli* emerging from the DEAE column. Fractions from the DEAE column containing top amount of protein and activity were combined and analyzed on SDS-PAGE (Figure S2).

Table S1. Summary of purification of PDC from yeast[a] and of yeast PDC overexpressed in *E. coli*.[b]

Step	Total activity, yeast, U	Total activity, *E. coli*, U	Total protein, yeast, mg	Total protein, *E. coli*, mg	Specific activity, yeast, U/mg	Specific activity, *E. coli*, U/mg	Recovery, yeast, %	Recovery, *E. coli*, %
crude extract	23,000	8,000	3,300	600	3	12	100	100
AS[c]	20,000	6,000	2,300	400	9	17	88	75
HT[d]	18,000	-	770	-	24	-	78	-
Dial.[e]	16,000	4,500	740	180	21	25	69	56
HTP[f]	9,900	-	330	-	30	-	42	-
DEAE[g]:								
1	1,400	30	27	1	52	30	6	0.3
2	700	304	16	8	44	38	3	3.8
3	250	585	6	13	42	45	1	7.3
4	1,600	1,000	34	20	47	50	7	12.5
5	410	688	11	16	37	43	2	8.6
6	530	400	13	10	41	40	2	5
7	240	96	8	3	30	32	1	1
total	5,100	3,103	115	71			22	39

[a]Data reproduced from (Farrenkopf & Jordan, 1992).

[b]Typical protocol for purification for 1 liter (4-5 g of cells) of scpdc1 overexpressed in *E. coli*

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[c]AS - ammonium sulfate precipitation; [d]HT - heat treatment; [e]Dial. - Dialysis; [f]HTP - hydroxyl apatite column; [g]DEAE - diethylamino ethyl Sepharose Fast Flow[R] fractions containing PDC activity.

Table S5. Kinetic data for WT PDC

pH	n	k_{cat} (s^{-1})	$S_{0.5}$ (mM)	$k_{cat}/S_{0.5}$ ($mM^{-1} s^{-1}$)	V_{max} ($mM\ min^{-1}$)	V_{max}/A ($mM^{-1} min^{-1}$)	V_{max}/B (min^{-1})
4.30	1.21						
4.50	1.31	7.73					
4.70		13.95		23.69			
5.00	1.55	26.82	1.06	25.28	13.98	84.92	
5.30	1.74	35.87	1.13	31.84	40.99	99.97	80.30
5.50	1.97	37.88	1.20	35.29	51.01	98.92	68.72
5.70	2.28	42.65	1.02	49.84	82.09	111.65	59.88
6.00	2.11	47.8	1.06	39.95	76.42	145.18	40.06
6.30	1.59	45.27	1.20	27.78	75.31	147.25	27.92
6.50	1.50	39.16	1.63	18.79	59.65	120.51	18.86
6.70	1.47	33.8	2.09	14.04	43.18	101.73	14.70
7.00	1.36	33.15	2.36	13.14		20.12	8.82
7.30	1.32	16.45	2.57	11.73		6.78	3.28
7.50		13.75		8.75			

Table S6. Kinetic data for the C221S PDC variant

pH	n	k_{cat} (s^{-1})	$S_{0.5}$ (mM)	$k_{cat}/S_{0.5}$ ($mM^{-1}s^{-1}$)	V_{max} ($mM\ min^{-1}$)	V_{max}/A ($mM^{-1}\ min^{-1}$)	V_{max}/B (min^{-1})
4.5		0.25		3.36			
4.70		0.90		2.09			
5.00	0.61	1.27	4.70		1.87	274.41	2.14
5.30	0.79	4.38	4.79	0.92	6.37	172.52	4.33
5.50	0.81	6.25	5.97	1.05	10.48	123.05	5.24
5.70	0.89	8.06	6.21	1.25	11.36	105.29	5.07
6.00	0.67	9.32	7.30	1.13	13.02	72.81	4.93
6.30	0.8	6.95			12.05	50.60	3.76
6.50	0.74	5.81	7.50	0.93	10.15	32.38	2.58
6.70	0.87	4.38	7.18	0.81	9.50	11.77	1.64
7.00	0.89	2.65	7.30	0.60	5.06	7.59	0.95
7.30		0.99		0.99	2.04	2.98	0.40
7.50		0.62			0.95	0.60	0.07

Table S7. Kinetic data for the H92G PDC variant

pH	n	k_{cat} (s^{-1})	$S_{0.5}$ (mM)	$k_{cat}/S_{0.5}$ ($mM^{-1} s^{-1}$)	V_{max} ($mM\ min^{-1}$)	V_{max}/A ($mM^{-1}\ min^{-1}$)	V_{max}/B (min^{-1})
4.50	1.29	3.28	4.32	0.75			
4.80	1.31	4.46	5.17	0.86			
5.00		5.42	3.20	1.70	4.64	1.15	2.21
5.30	1.3	8.81	4.61	1.91	9.14	3.94	
5.50	1.25	11.33	4.41	2.57	11.72	6.49	2.34
5.70	1.28	11.96	5.15	2.32	12.27	7.22	1.78
5.90	1.20	12.27	7.72	1.59	12.99		1.44
6.00	1.22	13.08	9.10	1.44	13.23		1.09
6.10	1.23	13.25	10.01	1.32	13.92	5.08	0.95
6.30	1.20	13.94	12.41	1.12	14.84	4.78	0.93
6.50	1.20	11.85	11.48	1.03	12.62	3.52	0.58
6.70	1.18	11.60	19.21	0.6	12.82	1.19	0.51
6.90	1.20	10.43	19.88	0.52	11.39	1.21	0.38
7.10	1.21	9.58	25.10	0.38	11.18	0.49	0.32
7.30	1.21	8.87	25.88	0.34	9.87		0.29
7.50	1.28	5.13	22.4	0.23	5.85	0.11	0.23

Table S8. Kinetic data for the H92K PDC variant

pH	n	k_{cat} (s^{-1})	$S_{0.5}$ (mM)	$k_{cat}/S_{0.5}$ ($mM^{-1} s^{-1}$)	V_{max} ($mM\ min^{-1}$)	V_{max}/A ($mM^{-1}\ min^{-1}$)	V_{max}/B (min^{-1})
5.07	1.44	27.94	0.34	82.59	28.70	673.18	136.74
5.50	1.53	25.79	0.58	44.64	26.19	152.30	97.75
5.70	1.55	26.09	0.80	32.54	28.16	143.21	77.11
5.90	1.45	30.49	0.72	42.35	30.97	114.40	77.06
6.02	1.49	28.72	0.84	40.78	29.26	102.13	58.58
6.30	1.53	28.42	1.22	25.13	28.91	38.20	48.81
6.52	1.53	28.57	1.34	21.36	29.11	32.21	43.80
6.70	1.49	27.16	2.16	12.60	27.79	13.58	22.60
6.91	1.36	26.04	2.45	10.61	26.78	15.74	14.81
7.10	1.34	22.04	3.25	6.77	22.77	8.34	8.98
7.30	1.40	22.24	5.03	4.42	23.31	7.04	4.88
7.50	1.42	15.60	5.25	2.97	16.34	1.83	4.15

Legends of Figures.

Figure S1. Elution profile of WT PDC from DEAE column. Activity (U/mL, - ■ -) and OD_{280} (solid line) are shown. The gradient is expressed as % of buffer B and is presented as a dashed line on the graph.

Figure S2. Analysis of WT PDC on SDS-PAGE: Lane 1 - molecular weight standards, molecular weights are shown in Da on the left hand side; Lane 2 - 1 μg of WT PDC.

Figures S6-S9. Plots of kinetic data listed in Tables S5-S8, respectively. The curves were calculated from a fit to the experimental points with the pK_{app}s listed in Table 9.

Figure S1. Baburina et al.

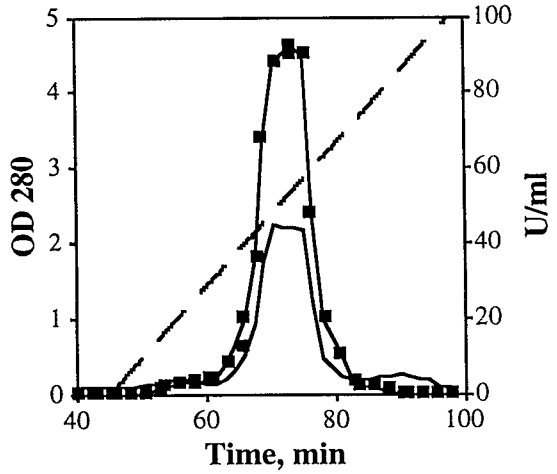

Figure S2. Baburina et al.

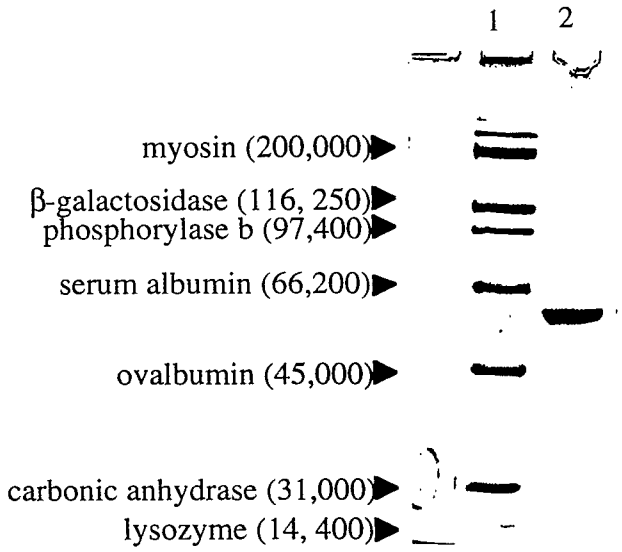

Figure S6. Baburina et al. WT PDC

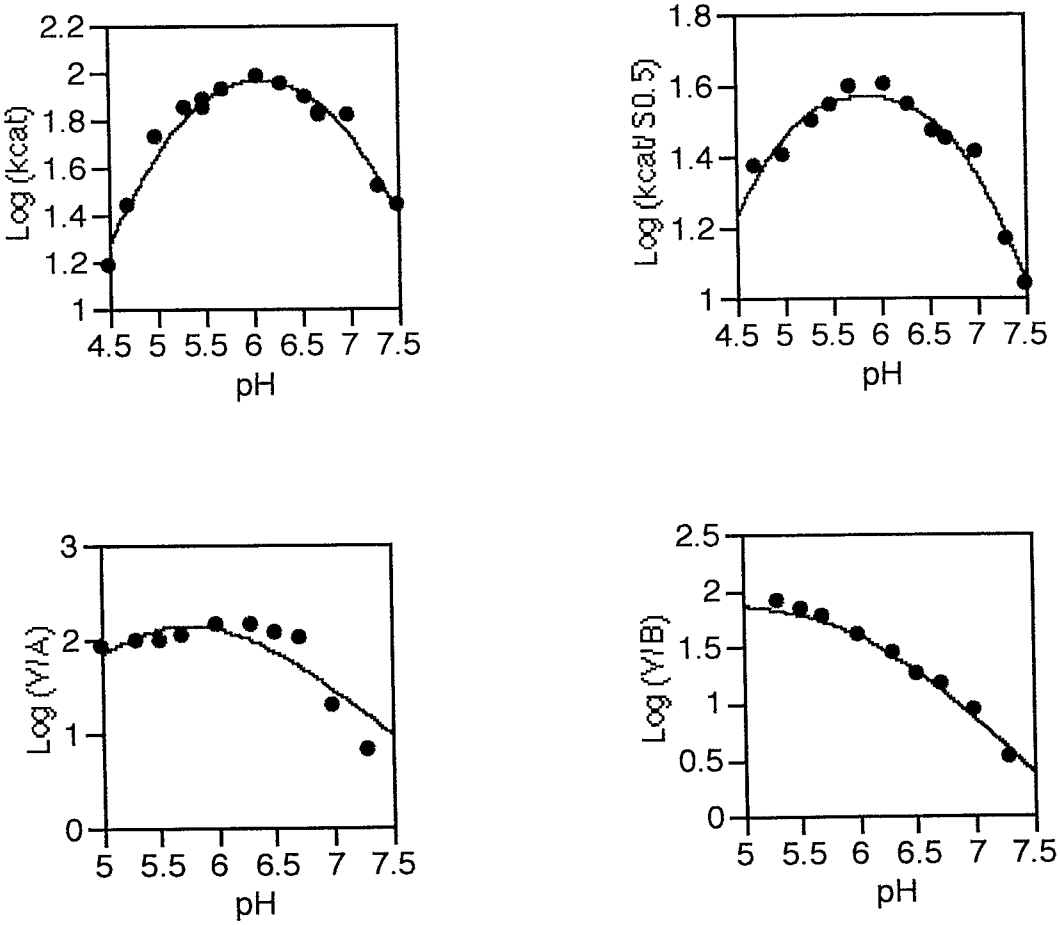

Figure S7. Baburina et al. C221S

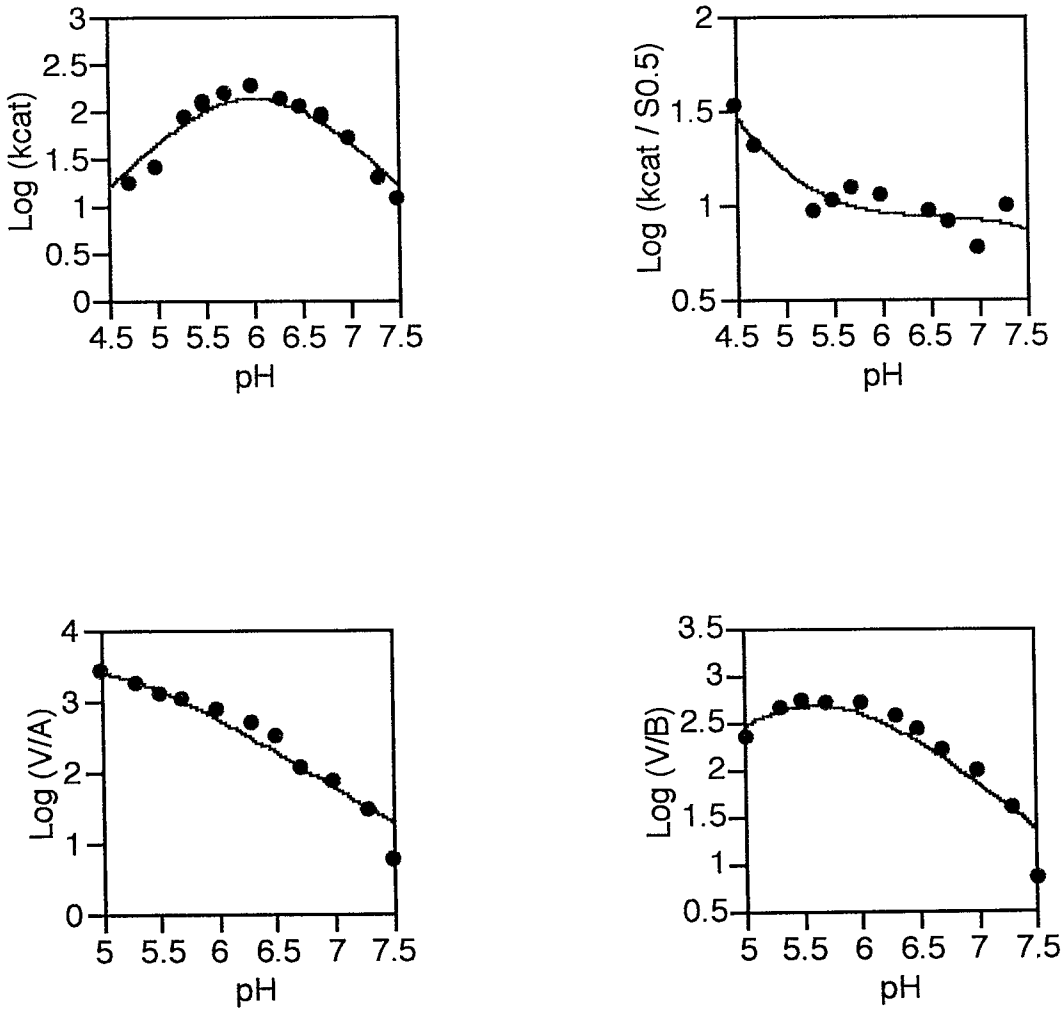

Figure S8. Baburina et al. H92G

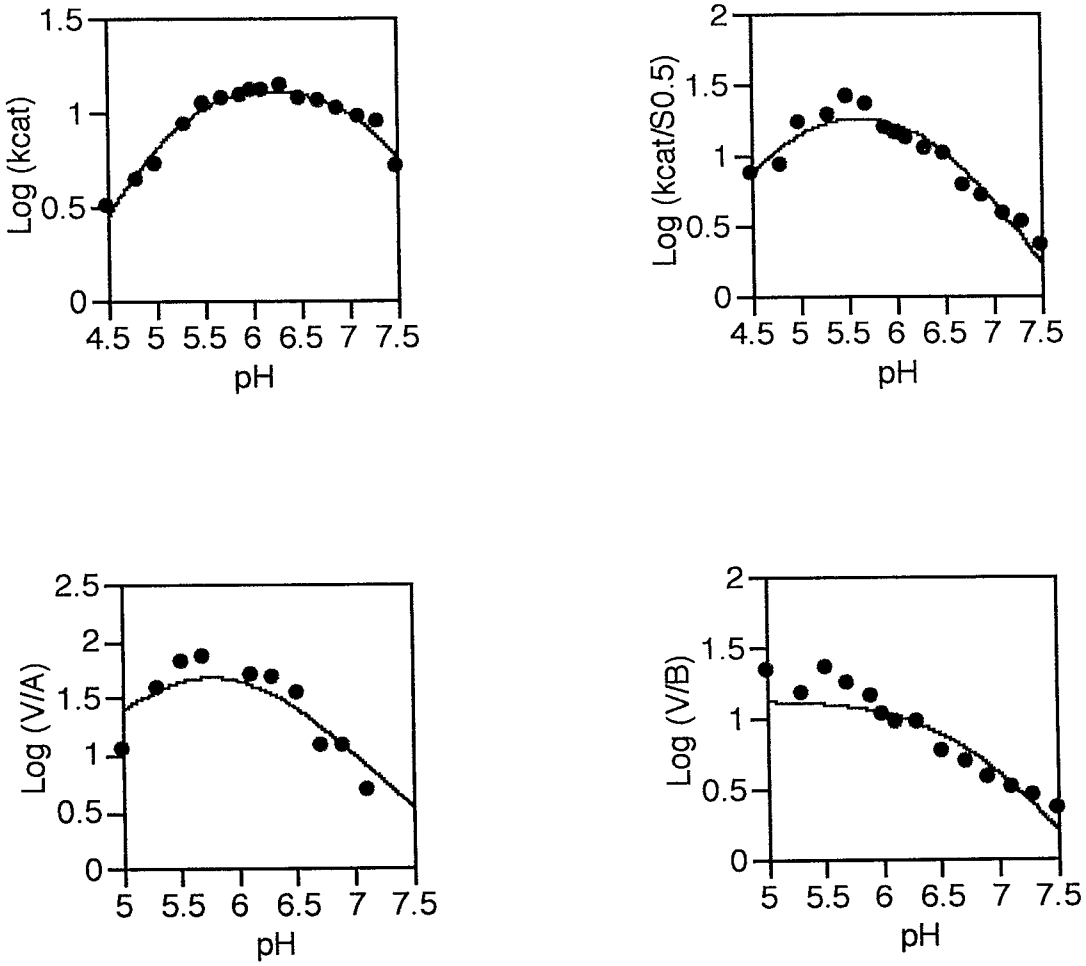

Figure S9. Baburina et al. H92K