Phenylalanyl Transfer Ribonucleic Acid Synthetase from Rat Liver. Analysis of Phenylalanine and Adenosine 5'-Triphosphate Binding Sites and Comparison to the Enzyme from *Escherichia coli*

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Inhibition of the ATP–PP_i exchange reaction catalyzed by rat liver phenylalanyl-tRNA synthetase by structural analogues of L-phenylalanine and ATP has been examined and compared with data reported for the enzyme from *E. coli*. The phenylalanine binding sites are similar in the following characteristics. (1) The region that complexes the phenyl ring shows a strict requirement for the unsubstituted phenyl ring although the rat liver enzyme is more tolerant in this respect. (2) The protonated amino group of phenylalanine is required for binding. (3) The region neighboring the binding site for the carboxylate of phenylalanine is diffusely hydrophobic. Unlike effects of these modifications on interaction with the *E. coli* enzyme, substitution of the carboxylate by hydrophobic groups leads to large losses in affinity for the rat liver enzyme. Although both enzymes bind D-phenylalanine very poorly, their relative affinities for the D isomers of phenylalanine analogues vary greatly. The most dramatic difference is observed with *N*-benzyl-D-amphetamine, which binds 24 000-fold tighter to *E. coli* phenylalanyl-tRNA synthetase than the rat liver enzyme. The affinity of rat liver phenylalanyl-tRNA synthetase for naturally occurring adenine compounds is similar to that of the *E. coli* enzyme, suggesting that the binding of ATP occurs via similar interactions. Adenine provides a major portion of the free energy of binding of ATP. The remainder may be viewed as the sum of detrimental interactions with the phosphate groups of ATP and a favorable contribution by the ribose moiety.

A number of investigations have been reported in which substrate analogues have been used to map the active sites of the aminoacyl-tRNA synthetases.[1-7] In previous studies from this laboratory, the interactions of analogues of L-phenylalanine and ATP with PRS from *Escherichia coli* have been examined in detail.[1-3] Here, we report a similar, albeit less extensive, analysis of the same enzyme from rat liver and compare the binding properties to the bacterial enzyme. These studies permit us to assign similarities in the substrate binding sites as well as large differences which may be exploited for the species-selective inhibition of PRS. Most interesting are analogues of *N*-benzyl-2-phenylethylamine which are extremely potent inhibitors of the bacterial enzyme but poor inhibitors of PRS from rat liver.

Experimental Section

Phe-tRNA synthetase was a 410-fold purified preparation obtained from Sprague–Dawley rat livers by modification of the method of Lanks et al.[8] Phenylmethylsulfonyl fluoride (0.5 mM) was included in the homogenization buffer, Sephadex G-25 was used for the gel filtration step, and the gradient elution step from phosphocellulose was omitted. Under conditions described by Lanks et al.[8] the protein catalyzed the formation of 18.5 nmol of Phe-tRNA per minute per milligram, corresponding to a purity of ca. 30%.[9] One unit of enzyme is that amount which will catalyze the ATP–PP_i exchange at a rate of 1 μmol/min under the standard conditions described below.

Carrier-free [^{32}P]PP_i and L-[^{3}H]phenylalanine (9.1 Ci/mmol) were purchased from New England Nuclear. Yeast tRNA from General Biochemicals was stripped prior to use by treatment with 1.8 M Tris–HCl (pH 8.0) for 20 min at 37 °C and subsequently desalted by passage through Sephadex G-25. ATP, L-phenylalanine, L-phenylalanineamide, *o*- and *m*-chlorophenylalanine, *m*-fluorophenylalanine, *o*-, *m*-, and *p*-methylphenylalanine, phenyl lactate, and phenyl pyruvate were products of Sigma Chemical Co. L-Amphetamine and 2-phenylethylamine were obtained from Aldrich Chemical Co. and D-phenylalanine was a product of Mann Research Laboratories. ADP and AMP were purchased from P-L Biochemicals and adenosine and adenine from Nutritional Biochemicals Corp. Dr. R. Abeles kindly donated 5'-deoxyadenosine. N-substituted L-phenylalanineamides, L-phenylalaninol, and *N*-methylphenylalanine were the preparations previously reported.[1] The *N*-benzyl derivatives of L-phenylalaninol, L-amphetamine, and 2-phenylethylamine will be described in the accompanying paper.[3]

ATP–PP_i Exchange Assay. For assaying phenylalanine analogues, the standard reaction mixture contained 2 mM ATP, 20 μM L-phenylalanine, 15 mM magnesium acetate, 1 mM [^{32}P]PP_i (ca. 2×10^5 cpm), 100 mM sodium cacodylate (pH 7.5), varying amounts of inhibitor, and a limiting amount of enzyme (ca. 5×10^{-4} unit) in a total volume of 0.25 ml. For assaying ATP analogues, ATP and phenylalanine concentrations in the above mixture were 0.25 and 0.20 mM, respectively. The mixtures were incubated at 37° and the amount of [^{32}P]ATP formed in 20 min was determined as previously reported.[10]

Analysis of Data. Approximate inhibition constants for the enzyme from the rat liver were obtained from plots of percent inhibition vs. log [inhibitor], using the equation $K_i = [I/S]_{50} \cdot K_m$ where $[I/S]_{50}$ is the ratio of inhibitor to substrate required for 50% inhibition of the initial velocity of exchange.

Results

Inhibition of the ATP–PP_i exchange reaction catalyzed by rat liver PRS has been examined using analogues of phenylalanine and ATP. Approximate values are listed in Table I along with the corresponding values which have been reported for *E. coli* PRS.[1,2]

ATP–PP_i Exchange Reaction Parameters. In order to optimize conditions for the ATP–PP_i exchange catalyzed by rat liver PRS, rates of reaction were determined as a function of pH and Mg^{2+} concentration. Maximal rates were achieved in 100 mM sodium cacodylate at pH 7.5 and in 100 mM Tris–HCl at pH 7.0. The optimal rate using cacodylate is 20% faster than the optimal rate in Tris–HCl. After correcting for the amount of Mg^{2+} complexed to ATP and PP_i, the optimal range and K_m for the free Mg^{2+} were determined to be 11–17 and 1.9 mM, respectively. These values are both slightly lower than those reported for the *E. coli* enzyme.[11] Michaelis constants (K_m) for L-phenylalanine and ATP in the ATP–PP_i exchange reaction were found to 0.020 and 0.25 mM, respectively. These values are very close to those reported[8] for the formation of Phe-tRNA.

Phenyl Ring. The area on *E. coli* PRS which complexes the phenyl ring of L-phenylalanine represents the primary site for recognition and one of the two major binding loci for the amino acid substrate. With the exception of fluorine, substituents invariably lead to loss of substrate activity and cause large decreases in binding energy.[1] These general observations also qualitatively describe the binding and substrate properties of ring-substituted phenylalanines using rat liver PRS; however, the losses in binding energy for these compounds relative

Table I. Inhibition of PRS-Catalyzed ATP-PP_i Exchange

	Compound	R	K_I, mM: Rat liver	K_I, mM: *E. coli*[a]	$\Delta\Delta F$, kcal/mol[b]
1	L-Phenylalanine (K_m)		0.020	0.050	−0.6
2	D-Phenylalanine		6.4	8.5	−0.2
3	L-Phenylalanol		0.80	0.0060	3.0
4	L-Amphetamine		0.70	0.012	2.5
5	D-Amphetamine		1.1	0.190	1.1
6	2-Phenethylamine		3.7	0.093	2.3
7	$RC_6H_4CH_2CH(NH_2)CO_2H$	*o*-CH_3	5.5	300[c]	−2.5
8	$RC_6H_4CH_2CH(NH_2)CO_2H$	*m*-CH_3	4.0	100[c]	−2.0
9	$RC_6H_4CH_2CH(NH_2)CO_2H$	*p*-CH_3	43[d]	*e*	
10	$RC_6H_4CH_2CH(NH_2)CO_2H$	*o*-Cl	7.5	*e*	
11	$RC_6H_4CH_2CH(NH_2)CO_2H$	*m*-Cl	7.5	23[c]	−0.7
12	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	H	0.33	0.035	1.4
13	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	CH_3	10[d]	30	−0.7
14	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	$(CH_2)_2CH_3$	0.70	2.46	−0.8
15	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	C_6H_5	1.3	0.20	1.2
16	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	$CH_2C_6H_5$	0.80	0.82	0.0
17	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	$(CH_2)_3C_6H_5$	0.56	0.068	1.3
18	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	$(CH_2)_4C_6H_5$	0.28	0.10	0.6
19	$C_6H_5CH_2CH(NH_2)C(=O)NHR$	$(CH_2)_5C_6H_5$	0.15[d]	0.045	0.7
20	$C_6H_5CH_2R$	$CH(OH)CO_2H$	11[d]	2.2	1.0
21	$C_6H_5CH_2R$	$C(=O)CO_2H$	10[d]	7.6	0.2
22	$C_6H_5CH_2R$	$CH(NCH_3)CO_2H$	36[d]	18	0.4
23	$C_6H_5CH(NHCH_2C_6H_5)R$	L-CH_2OH	3.0	0.038[f]	2.7
24	$C_6H_5CH(NHCH_2C_6H_5)R$	L-CH_3	3.7	0.0056[f]	4.0
25	$C_6H_5CH(NHCH_2C_6H_5)R$	D-CH_3	3.4	0.00014[f]	6.2
26	$C_6H_5CH(NHCH_2C_6H_5)R$	H	3.7	0.00067[f]	5.3
27	ATP (K_m)[g]		0.25	0.80	−0.7
28	ADP[g]		1.3[d]	2.4	−0.4
29	AMP		4.4[d]	3.7	0.1
30	Adenosine		0.066	0.015	0.9
31	5′-Deoxyadenosine		0.045	0.001	2.3
32	Adenine		0.55	0.50	0.1

[a] Data from ref 1 and 2. [b] Calculated from ΔF (rat liver) − (*E. coli*). [c] Estimated from percent inhibition at 12 mM. [d] Value extrapolated from the percent inhibition observed at maximum solubility. [e] No inhibition at 12 mM. [f] Data from ref 3. [g] Added to reaction mixtures as the magnesium salt.

to L-phenylalanine are substantially lower than observed with the *E. coli* enzyme. *E. coli* PRS also appears to tolerate meta better than ortho substituents, whereas the rat liver exhibits approximately the same affinity for both ortho- and meta-substituted phenylalanines; para substituents are the most detrimental for binding to either synthetase. Under assay conditions in which phenylalanine was omitted, only *m*-fluorophenylalanine stimulated rat liver PRS catalyzed ATP-PP_i exchange.

α-Carboxylate Region. With the *E. coli* activating enzyme, the α-carboxyl of L-phenylalanine is not necessary for optimal binding, and its substitution by hydrophobic groups leads to substantial increases in affinity for the enzyme.[1,3] This has been interpreted as evidence that this region of the enzyme is partially hydrophobic. The data obtained using rat liver PRS suggest that the carboxylate binding region is significantly different. Omission of the carboxylate of L-phenylalanine to give 2-phenylethylamine (**6**) has little effect on binding to *E. coli* PRS but results in a dramatic decrease (3.2 kcal/mol) in affinity for the rat liver enzyme. An increase of ca. 1–1.5 kcal/mol of binding to rat liver PRS is observed when methyl (**4**), hydroxymethyl (**3**), and carboxamide (**12**) groups are added to 2-phenylethylamine in the position occupied by the carbonyl of L-phenylalanine, but none of these bind as well as the substrate. In contrast, the same compounds all bind tighter than L-phenylalanine to the *E. coli* enzyme; they complex to *E. coli* PRS with ca. 2.5–3 kcal/mol greater free energy of binding than to rat liver PRS. Contrasting with *E. coli* PRS, it seems likely that the carboxylate of L-phenylalanine contributes to binding to rat liver PRS. Other substituents—hydroxymethyl (**3**), methyl (**4**), or carboxamide (**12**)—also contribute albeit not as strongly. Since these groups are quite hydrophobic compared to the solvated carboxyl group of the substrate, it is reasonable to speculate that the carboxylate binding region on rat liver PRS is in general hydrophilic but that the microenvironment of this site is such that hydrophobic groups can also be tolerated and contribute to binding to a small extent.

The overall pattern of binding of phenylalanine alkyl- and phenylalkylamide to the rat liver and *E. coli* enzymes suggests that the regions on these enzymes which extend from the carboxylate of bound phenylalanine are quite similar. Attachment of an *N*-methyl group to phenylalanineamide (**12**) to give **13** leads to a 4.2 kcal/mol loss in binding energy to *E. coli* PRS which is attributed to a very unfavorable steric interaction. A similar but smaller loss, 2.1 kcal/mol, is observed for phenylalanine *N*-methylamide binding to rat liver PRS, indicating that this enzyme exhibits less steric hindrance at this point than the *E. coli* enzyme. A phenyl group is also detrimental to binding, but less so than a methyl group. As observed for the *E. coli* enzyme, this detrimental steric effect may be overcome by favorable compensatory interactions obtained upon extending the carbon chain of alkyl- (**14**) and phenylalkylamides (**17**–**19**) of L-phenylalanine. Although the average increase in binding energy per methylene group (0.33 kcal/mol) is less than the 0.8–1.0 kcal/mol which is expected for maximal hydrophobic interaction, sufficient binding energy is attained such that phenylpentylamide (**19**) binds ca. twofold better than phenylalanineamide (**12**) and 60-fold better than the *N*-methylamide (**13**) to rat liver PRS. The data support the notion that a hydrophobic

region is neighboring to the carboxyl binding site of both the rat liver and *E. coli* enzymes.

Stereospecificity. Rat liver PRS shows high specificity for the L isomer of phenylalanine. As with the *E. coli* enzyme, D-phenylalanine is not a substrate for rat liver PRS and is a poor inhibitor; relative to L-phenylalanine, the loss in energy of binding to either synthetase is greater than 3 kcal/mol. Large differences are also observed in binding of the L and D isomers of the phenylalanine analogues (**3–6**, **12**) and their *N*-benzyl derivatives (**23–26**) to *E. coli* PRS. In contrast, L- and D-amphetamine (**4**, **5**), as well as their *N*-benzyl derivatives (**25**, **26**), exhibit about the same affinity for rat liver PRS as for the bacterial PRS.

α-Amino Group. As observed for a number of other aminoacyl-tRNA synthetases[4,5,12] the α-amino group is necessary for the binding of phenylalanine by rat liver PRS. Replacement of the α-amino group by hydroxy (**20**) or oxo (**21**) leads to a 2.5 kcal/mol loss in binding energy. *N*-Methylphenylalanine (**22**) binds approximately 3.2 kcal/mol poorer than L-phenylalanine to rat liver PRS; this loss in binding is presumed to be the result of a highly unfavorable steric interaction of the methyl group. These results are similar to those observed for *E. coli* PRS.[1]

Addition of an *N*-benzyl group to L-amphetamine or L-phenylalaninol does not cause a dramatic change in the affinity of these compounds for *E. coli* PRS, whereas *N*-benzyl-D-amphetamine (**25**) and *N*-benzyl-2-phenylethylamine (**26**) are the most potent inhibitors known for the bacterial enzyme.[3] The reason for the potency of these inhibitors is not clear at this time. In contrast, the *N*-benzylphenylalanine analogues **23–26** all bind to rat liver PRS with a $K_i \simeq 3$ mM, regardless of stereochemistry. Thus, *N*-benzyl-2-phenylethylamine and *N*-benzyl-D-amphetamine show 5300- and 24 000-fold higher affinities for the *E. coli* enzyme than rat liver PRS.

Analogues of ATP. In contrast with the marked differences between the phenylalanine binding sites of the *E. coli* and rat liver enzymes, the manner in which these enzymes bind ATP and related compounds is, in general, quite similar. The 6-aminopurine moiety of ATP contributes the major portion of binding energy since adenine (**32**) binds to rat liver PRS only twofold worse than ATP (**27**). As with the *E. coli* enzyme, the remainder of the interactions of ATP with enzyme may be described as a balance between beneficial and detrimental interactions occurring at other points on the substrate. For rat liver PRS, addition of a ribose ring to adenine to give adenosine (**30**) results in an increase in binding of 1.3 kcal/mol. Addition of a phosphate group to adenosine to give AMP (**29**) causes a 2.6 kcal/mol decrease in binding. Further addition of phosphate groups to give ADP (**28**) and ATP (**27**) leads to a progressive increase in binding energy; however, as observed for *E. coli* PRS, this increase in binding energy is not sufficient to overcome the detrimental interaction imposed by addition of the first phosphate group.[2] Since adenosine binds ca. fourfold better than ATP to rat liver PRS, it is concluded that the phosphate groups of ATP do not provide a significant contribution to binding. The ATP binding sites of the two enzymes appear to differ somewhat in the region neighboring the 5′-methylene group. 5′-Deoxyadenosine (**31**) binds 15-fold tighter than adenosine (**30**) to *E. coli* PRS[2] whereas these two compounds exhibit similar affinities for the rat liver enzyme.

Discussion

Optimal interactions within an enzyme–substrate complex require that regions of the enzyme complement the binding points of the substrate in topography, polarity, charge, and solvation. Analysis of the binding properties of structural analogues of a substrate provides an approach to probing these features in a manner which is not amenable to other physical measurements. In addition, a study of structural analogues often uncovers information about regions surrounding the active site which may be useful in the design of potent and specific enzyme inhibitors and in comparative studies which seek to uncover subtle differences among related enzymes.[13]

In the studies described here, the phenylalanyl-tRNA synthetases obtained from *E. coli* and rat liver are compared with regard to their affinities for a variety of phenylalanine and ATP analogues. *A priori*, one might expect binding regions critical to specificity and catalysis to be quite similar since the same substrates and catalytic reaction are involved. In addition, the phenylalanyl-tRNA synthetases from *E. coli*,[14] yeast,[15] and rat liver[9] have similar molecular weights (ca. 270 000) and an $\alpha_2\beta_2$ subunit structure which is somewhat unusual among this class of enzymes.[16] From this, it would appear that many characteristics of PRS have been conserved through evolution. Although the critical binding sites required for substrate specificity and catalysis are likely to be conserved, regions outside the active site could show variation with little effect on the catalytic reaction or physical properties of the proteins. Baker[13] has suggested that species variation in substrate identical enzymes might best be detected by searching for differences in affinities for substrate analogues. The objective of this study was to ascertain whether significant species differences existed in phenylalanyl-tRNA synthetase which might be exploitable using classical and nonclassical[13] inhibitors.

Detailed interpretations of the mode of binding of the analogues examined here are provided in the previous section and reports.[1-3] As expected, most regions critical to binding of substrates appear to be similar in the *E. coli* and rat liver phenylalanyl-tRNA synthetases. Only minor differences are detectable in (1) the regions which bind the phenyl and amino groups of phenylalanine, (2) the area adjacent to and extending from the carboxylate binding region of phenylalanine, (3) the stereospecific recognition of L-phenylalanine, and (4) the ATP binding regions. However, there are dramatic differences in at least two regions of the enzyme from *E. coli* and rat liver which are readily detectable by the differential inhibition by phenylalanine analogues.

The first region is that which binds the carboxylate moiety of L-phenylalanine. With the *E. coli* enzyme, omission of the carboxylate of L-phenylalanine to give 2-phenylethylamine does not result in a significant decrease in binding. Moreover, substitution of the carboxylate by comparatively hydrophobic groups (CH_2OH and CH_3) results in significant increases in binding. This and other observations have supported the notion that this binding region of *E. coli* PRS is hydrophobic in nature and the hydrated carboxylate of phenylalanine is partially desolvated upon binding. In addition, the *E. coli* enzyme retains its requirements for stereospecificity in binding to these analogues.[1] In striking contrast, these analogues are poor inhibitors of the rat liver PRS and the enzyme shows little or no stereospecific discrimination. We have concluded that the carboxylate binding region of the rat liver enzyme is, in general, hydrophilic but may possess a hydrophobic microenvironment which permits minimal interaction with the hydrophobic constituents of these analogues. It should be emphasized that the differences in binding of these analogues to the two enzymes are not trivial; one of these (L-phenylalanol) binds over 100-fold

tighter to the *E. coli* enzyme.

The other region of these two enzymes which may be differentiated with inhibitors is that which extends from the α-amino group. *N*-Benzyl-2-phenylethylamine and its α-substituted analogues are the most potent inhibitors of the *E. coli* PRS yet reported (K_i = 10^{-6}–10^{-7} M).[3] In contrast, these analogues are very poor inhibitors of the rat liver enzyme. For example, the dissociation constants for *N*-benzyl-D-amphetamine differ by a factor of 24 000 ($\Delta\Delta F$ = 6.2 kcal/mol). From these comparisons, it follows that such inhibitors would completely inhibit the bacterial enzyme at concentrations which would not effect the mammalian PRS. Since this enzyme is so critical for protein synthesis, these analogues might be of use as potent and selective chemotherapeutic agents. Studies in this laboratory (unpublished results) have demonstrated that the growth of *E. coli* is inhibited by such analogues as a direct result of specific in vivo inhibition of PRS.

Lastly, it should be noted that these studies provide another illustration of the feasibility of approaching drug design at a molecular level. An enzyme was chosen which was critical to cellular function and its substrate binding properties were examined in detail.[1,2] From these results, analogues were designed[3] which were sufficiently potent that they were selective for the target enzyme in vivo. It has now been demonstrated that some of these compounds, notably nonclassical inhibitors,[13] also show a dramatic species selectivity. It remains to be demonstrated that such compounds will be useful chemotherapeutic agents and such studies shall be performed in the near future.

Acknowledgment. This work was supported by U.S. Public Health Service Grant CA-14266 from the National Cancer Institute. D.V.S. is the recipient of a U.S. Public Health Service Research Career Development Award from the National Cancer Institute.

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Evidence for Species-Specific Substrate-Site-Directed Inactivation of Rabbit Adenylate Kinase by N^6-(6-Iodoacetamido-*n*-hexyl)adenosine 5'-Triphosphate†

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Received January 12, 1976

Adenosine 5'-triphosphate (ATP) derivatives bearing iodoacetylamino-*n*-alkyl substituents [$(CH_2)_nNHCOCH_2I$] on N^6 were synthesized as potential ATP-site-directed irreversible inhibitors of adenylate kinases from rabbit, pig, and carp muscle. When *n* was 5 no enzyme was progressively inhibited (inactivated) by 1 mM inhibitor under the test conditions (6 h at 0°); when *n* was 6 the rabbit enzyme was 76% inactivated by 0.79 mM inhibitor whereas the pig and carp enzymes were unaffected by 2.76 mM inhibitor; when *n* was 7, 1 mM inhibitor inactivated 14% of the rabbit enzyme and did not inactivate the pig and carp enzymes; when *n* was 8, all enzymes were inactivated 11–15% by 1 mM inhibitor. No inactivation occurred when the iodine of the hexamethylene analogue was replaced by hydrogen. The selective effect occurred also in mixtures of the rabbit and pig enzymes and evidence could not be found that the hexamethylene analogue was activated by the rabbit enzyme or deactivated by the pig and carp preparations. The species-specific inactivation is concluded from various lines of evidence to be ATP-site-directed and is attributed to alkylation of an amino acid residue of the rabbit enzyme which in the pig and carp enzymes is absent, inaccessible, or less reactive. These and previous studies with several other enzymes provide evidence that substrate-site-directed agents capable of bonding covalently to an amino acid residue outside the substrate site can be designed to exert species-specific or tissue-specific irreversible inhibition of target enzymes.

In the course of work on the systematic development of substrate-site-directed exo-site enzyme reagents[1] Baker and co-workers reported several instances in which this approach produced tissue-selective[2,3] or species-selective[4] irreversible enzyme inhibitors. We now describe a second example of species selectivity in which a substrate derivative inactivates adenylate kinase of rabbit muscle and at a higher concentration does not inactivate the same enzyme from pig or carp muscle. These enzymes catalyze phosphoryl transfer from adenosine 5'-triphosphate (ATP) to adenosine 5'-phosphate (AMP) to produce adenosine 5'-diphosphate (ADP). As candidate irreversible inhibitors N^6-alkyl derivatives of ATP (structure **3**) were selected because previous work with N^6-Bz-ATP derivatives[5] had shown that the enzyme–ATP complexes of the above three kinases have extensive bulk tolerance near N^6 of ATP. A brief account of most of the present findings has been presented.[6]

† This paper is dedicated to the memory of the late Bernard R. Baker.