Molecular Physiology of Phosphoryl Group Transfer from Carbamoyl Phosphate by a Hyperthermophilic Enzyme at Low Temperature†

Santiago Ramón-Maiques, Hubert G. Britton, and Vicente Rubio*

Instituto de Biomedicina de Valencia (CSIC), Jaume Roig 11, 46010-Valencia, Spain

Received August 9, 2001; Revised Manuscript Received December 14, 2001

ABSTRACT: Enzymes from thermophilic organisms often exhibit low activity at reduced temperature. To obtain a better understanding of this sluggishness, we have studied the reaction at 24 °C of the carbamate kinase (CK) from the hyperthermophile *Pyrococcus furiosus*. This enzyme is much slower at low temperature than is the CK from the mesophile *Enterococcus faecalis*. X-ray structures demonstrated bound ADP (even when no nucleotide was added) with the hyperthermophilic but not with the mesophilic CK. We use centrifugal gel filtration, rate of dialysis and pulse−chase experiments to demonstrate that the pyrococcal enzyme, at 24 °C, binds ADP avidly ($K_D = 34$ nM), that ADP dissociates from this complex with a $t_{1/2}$ value of 2.4 s, and that ADP binding is very fast ($k = 8.4 \times 10^6$ M^{-1} s^{-1}). The high affinity, rather than restrictions to dissociation, explains the isolation of the pyrococcal enzyme as an ADP complex. Carbamoyl phosphate adds quickly to this complex, and ADP cannot dissociate from the resulting ternary complex, being that it is converted very slowly ($t_{1/2} = 10.3$ s) to ATP, which dissociates quickly ($t_{1/2} <$ 2.4 s). The slow conversion is a part of the normal enzyme reaction and limits the rate of the reaction at 24 °C. Thus, the sluggishness of the enzyme at low temperature is not due to slow substrate binding or product release but to the very slow rate of isomerization between enzyme-bound substrates and products. Probably the catalysis of the phosphoryl group transfer is less efficient at low temperature, as suggested by structural data showing that Lys131 is improperly positioned to assist the transfer.

The enzymes from thermophilic organisms differ from those found in organisms living at more normal temperatures not only in their high thermal stability (*1*, *2*), but generally also in their being much slower catalysts than are their mesophilic counterparts (*3*, *4*). It is frequently observed that the rate of catalysis is similar for the thermophilic and mesophilic enzymes at the characteristic temperatures at which their corresponding organisms generally live (*3*). We are beginning to understand the factors that render thermophilic enzymes highly resistant to heat, particularly the existence of ion pair networks (5) and extended hydrophobic interactions (*6*) in addition to, in some cases, the clustering of oligomers (*7*). However, the reasons that underlie the low catalytic efficiency of these enzymes at normal temperatures remain to be determined, although it is common to invoke the lack of flexibility of protein regions at relatively low temperatures as a possible cause for the decreased catalytic efficiency (*8*, *9*). In the case of indoleglycerol phosphate synthetase, a reduced rate of product dissociation was found and appeared to justify the decreased rate (*4*), although it is presently unknown if this observation can be applied to the generality of thermophilic enzymes.

A particularly good model to study the reasons that cause the low catalytic efficiency of thermophilic enzymes is provided by the carbamate kinase from *Pyrococcus furiosus* (*10*). This enzyme exhibits an unsually high thermal stability, (it survives being boiled for extended periods of time (*11*)), whereas its mesophilic counterpart, the carbamate kinase from *Enterococcus faecalis*, is destroyed by heating for 10 min at 55 °C (*12*). In addition, the hyperthermophilic enzyme catalyzes the reaction at mesophilic temperatures (25−37 °C) 2−3 orders of magnitude more slowly than does the enterococcal enzyme (*13*). Both enzymes exhibit 50% sequence identity (*11*, *12*), and their 3-D structures have been found by X-ray crystallography to be vitually identical (*14*), consisting of homodimers with an independent active center within each subunit. An important difference between the two enzymes, as was found in the crystal structure of the pyrococcal enzyme, is a bound ADP[1] substrate molecule in the enzyme active center (*14*), whereas no nucleotide was found associated with the crystalline mesophilic enzyme (*15*). The nucleotide was found in the pyrococcal enzyme crystals irrespective of whether the enzyme was exposed to this nucleotide during the purification and crystallization processes, and it was concluded that the ADP was or was not derived from the *Escherichia coli* cells in which the enzyme was produced and remains bound to the enzyme through the entire experimental procedure lasting several weeks (*14*). On

† This work was supported by grants from the Fundación Ramón Areces and PM97-0134-C02-01 from the Dirección General de Enseñanza Superior (DGES). S.R.-M. was a Predoctoral Fellow and H.G.B. was a recipient of a bursary for visiting scientists of the Generalitat Valenciana.

* Corresponding author. E-mail: rubio@ibv.csic.es. Tel: +34-96-3391772. Fax: +34-96-3690800,

[1] Abbreviations: CK, carbamate kinase; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; K_D, dissociation constant; $t_{1/2}$, half-life; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TLC, thin-layer chromatography; carbamoyl-P or CP, carbamoyl phosphate.

10.1021/bi011637d CCC: $22.00
Published on Web 03/03/2002

the basis of this finding, it was hypothesized that ADP might dissociate very slowly from the enzyme and that the slow dissociation of the nucleotide might limit the rate of the reaction in this hyperthermophilic carbamate kinase (*14*), as had been reported for the products of thermophilic indoleglycerol phosphate synthase (*4*).

We now test the above possibility in mechanistic studies of *P. furiosus* CK using pulse−chase and binding studies. A surprising conclusion of these studies is that in *P. furiosus* CK, bound ADP is able to exchange at a reasonably rapid rate with ADP in the solution. Therefore, finding ADP bound to the enzyme is not the consequence of sequestration of the nucleotide. The studies also demonstrate an extremely low K_D value for ADP of the pyrococcal enzyme and show that the high affinity rather than the slow rate of dissociation is the reason that the enzyme is isolated and crystallized as a complex with ADP.

Our work has also revealed that, rather than being limited by the binding of the substrates or the dissociation of the products, the velocity of the reaction of ATP synthesis at low temperature is limited by the isomerization step between the enzyme−substrates complex and the enzyme−products complex, which possibly can be equated to slowed catalysis of the phosphoryl group transfer process. Structural insight into the possible mechanism for this reduced catalytic efficiency is provided.

MATERIALS AND METHODS

Chemicals and General Conditions. [8-^{14}C]ADP (55 Ci/mol, 55 μCi/mL) and Sephadex G-50 (fine) were from Amersham-Pharmacia. PEI-cellulose plastic TLC sheets with fluorescent indicator (DC-Plastikfolien PEI-Cellulose F) were from Merck. The scintillation fluid was OptiPhase Hisafe II (from EG&G). The Bradford reagent (*16*) for protein determination was obtained from BioRad. ADP (disodium salt) was of the highest quality from Sigma. The concentration of the nucleotide was determined from its optical absorption at 259 nm, at neutral pH, using an absorption coefficient of 15.4 cm^{-1} mM^{-1} (*17*). Other reagents were of the highest quality available from Merck, Roche Diagnostics, or Sigma.

Unless indicated, all procedures were carried out at 24 °C in buffer A, consisting of a solution of 50 mM Tris/HCl (pH 8), 50 mM KCl, 10 mM $MgCl_2$, and 0.02 % sodium azide.

Enzymes. Recombinant carbamate kinase (previously called carbamate kinase-like carbamoyl phosphate synthetase (*11*, *13*, *14*)) from *P. furiosus* was overexpressed in *E. coli* and was purified exactly as described (*10*, *13*). The enzyme was placed in buffer A by repeated cycles of dilution and concentration using centrifugal ultrafiltration (Microsep Centrifugal Concentrators of 10 K cutoff, from Pall-Filtron). Enzyme concentration is given as the concentration of the carbamate kinase polypeptide (mass = 34.3 kDa). Pyruvate kinase (from rabbit muscle, salt-free) and lactate dehydrogenase (from hog muscle, salt-free) were from Roche Diagnostics.

Determination of ADP Content of Purified CK. Equal volumes of a solution of the enzyme (6.9 mg/mL) in buffer A and of 0.3 M $HClO_4$ were mixed and held at 0 °C for 10 min. Precipitated protein was removed by centrifugation at 4 °C, and the supernatant was neutralized with 3 M Tris base. Then 50 μL of the neutralized solution was added to a 1-mL spectrophotomer cuvette containing 0.8 mL of a solution at 37 °C of 50 mM glycylglycine (pH 7.4), 15 mM $MgSO_4$, 50 mM KCl, 2.5 mM phosphoenolpyruvate, 5 mM β-mercaptoethanol, and 0.25 mM NADH. The optical density at 340 nm was determined, and the decrease in the absorption was monitored after addition of 2 μL of a mixture of pyruvate kinase and lactate dehydrogenase (5 mg/mL each). An absorption coefficient of 6.22 cm^{-1} mM^{-1} for NADH (*18*) was used.

Centrifugal Gel Filtration. The assays were carried out exactly as described (*19*), using Sephadex G-50 (fine) equilibrated in buffer A and placed in 1-mL tuberculin syringes to remove unbound low-molecular-weight compounds rapidly. These compounds are retained in the column from the protein and protein-bound ligands that are excluded and appear in the effluent. Samples of 0.05 mL were applied to the columns, and the centrifugation was started immediately. When indicated, column slicing into four sections and determination of the radioactivity in each section was carried out (*20*).

ADP Binding Assays Using the Rate of Dialysis. The assays were based on the principles of flow dialysis (*21*). Care was taken to ensure a constant geometry and rate of stirring in the different dialysis steps. One milliliter of a solution of 1 mg/mL of the enzyme in buffer A was mixed with a trace of [^{14}C]ADP (180 000 cpm) and was placed in a dialysis bag (flat width, 1 cm) in a 250-mL cylinder. Dialysis was carried out with brisk magnetic stirring against 120 mL of external buffer A and with monitoring in the external fluid the time course of radioactivity exit and by estimating the internal radioactivity by difference. The external fluid was replaced with fresh fluid when >10% of the radioactivity had come out. The whole process was repeated 4−5 times (approximately 80 h total), and then the dialysis bag was opened to assay its protein and radioactive contents and to add 365 nmol of unlabeled ADP. A sample of the bag contents was used at this stage to confirm that the radioactivity corresponded to ADP by using PEI-cellulose chromatography (see below). The bag was closed, and dialysis was carried out again versus fresh buffer by monitoring the time-course of the outflow of the radioactivity, which this time was very fast, reaching equilibrium within 6 h. The amount of protein and the enzyme activity within the dialysis bag were the same at the beginning and at the end of the experiment. The proportion of the internal ADP that was free at each buffer change was estimated from the ratio of the initial rate of exit of radioactivity (as a fraction of the total radioactivity inside the bag) to the initial rate of exit observed after the final addition of excess unlabeled ADP. The amount of internal ADP at each buffer change was estimated from the radioactivity remaining in the bag and was based on the ADP content of the initial enzyme solution of 0.93 mol ADP/mol enzyme.

Pulse−Chase Experiments. The enzyme, labeled by incubation with a trace of [^{14}C]ADP, was mixed with a solution containing carbamoyl phosphate and excess unlabeled ADP. After a certain time, the reaction was terminated with acid, and the radioactivity in ATP and ADP was determined after separation of the nucleotides using PEI-cellulose thin layer sheets (10-cm) (*22*, *23*), which were developed with 0.5 M

Table 1: Labeling of the Enzyme upon Incubation with [8-^{14}C]ADP[a]

experiment	[^{14}C]ADP added, cpm/mL ($\times 10^3$)	unlabeled ADP added, mM: 1st incubation	unlabeled ADP added, mM: 2nd incubation	radioactivity in effluent, cpm/mg protein (mol/mol enzyme)
I	411			138 000
	402	3.86		2000
II	492	0.61		17 093 (0.80)[b]
	492	0.61	4.60	2576 (0.95)[b]

[a] The enzyme (60 μM) in buffer A was incubated in experiment I for 5 min with a trace of radioactive ADP and when indicated, with the concentration of unlabeled ADP specified. In experiment II, the enzyme was first incubated 1 min with an approximately 10-fold molar excess of ADP containing the indicated amount of radioactivity. Then, when indicated, the amount shown of unlabeled ADP was added, and the incubation was continued for 1 additional min (2nd incubation). Incubations were terminated by subjecting 50 μL samples to centrifugal gel filtration through 1 mL columns equilibrated in buffer A, followed by the determination of protein and radioactivity in the effluent. [b] Estimated by taking into account the calculation of the specific activity of the amount of ADP bound initially to the enzyme preparation (0.93 mol/mol enzyme).

phosphoric acid brought to pH 3.5 with KOH (*24*). The radioactivity incorporated into ATP was expressed as a fraction of that found in ADP + ATP. Details and concentrations are given with each assay in the captions to the Tables and Figures.

Other Assays. Enzyme activity in the direction of carbamoyl phosphate synthesis was determined at 37 °C by coupling with ornithine transcarbamylase (*10*). Protein concentration was quantitated by the method of Bradford (*16*), using bovine serum albumin as a standard. The program GraphPadPrism was used for curve fitting and statistical evaluation.

RESULTS

ADP Content of the Enzyme. In previous work, X-ray diffraction of enzyme crystals grown in the absence of a nucleotide showed the presence, with essentially full occupancy, of a well-defined ADP molecule per enzyme subunit (*14*). To quantitate this occupancy by the enzymatic assay of ADP, the enzyme was transferred to buffer A by repeated cycles of concentration and redilution using a centrifugal ultrafilter to remove any ADP that might be in solution, and then the protein was acid-denatured and ADP was assayed spectrophotometrically in the neutralized supernatant using an NADH-based coupled assay. A mean value of 187.5 nmol of ADP/mL of a 6.9 mg/mL solution of the enzyme was obtained, corresponding to 0.93 mol of ADP/mol of enzyme monomer. No ADP was detected in any of the samples of suspending buffer.

Enzyme-Bound ADP Exchanges with ADP in Solution. Because the enzyme had undergone repeated cycles of ultrafiltration and dilution, the persistence of bound ADP might reflect irreversible binding. However, when the enzyme was incubated for 5 min with a trace of [8-^{14}C]ADP and then the mixture was subjected to centrifugal gel filtration to remove unbound ADP (which is retained by the column), a large amount of radioactivity appeared in the column effluent, as expected if the radioactive ADP had become bound to the enzyme (Table 1, experiment I). In contrast, little radioactivity appeared in the effluent if a large excess (relative, in molar terms, to the amount of enzyme) of unlabeled ADP was also added to the mixture, as expected if the majority of the nucleotide remained unbound and was retained by the column. The simplest interpretation of these results is that during the 5 min incubation period the unlabeled ADP initially bound to the enzyme equilibrates with the [^{14}C]ADP added, and thus the enzyme−ADP complex becomes labeled. However, because there is a small but substantial fraction (7%) of the enzyme molecules that are devoid of ADP, the labeling could be due to the binding of the exogenously added [^{14}C]ADP to the free enzyme molecules, without dissociation of the initially bound ADP and equilibration with the ADP in the solution. To differentiate between these alternatives, experiment II (listed in Table 1), in which a 10-fold molar excess (with respect to the enzyme monomer) of [^{14}C]ADP was added to the enzyme to minimize the influence of the enzyme-bound ADP on the specific activity of the nucleotide, was performed. After a short incubation, the solution was subjected to centrifugal gel filtration. The radioactivity in the effluent greatly exceeded that expected if only 7% of the enzyme molecules originally devoid of ADP was labeled; rather, it corresponded to a value not far from 1 mol ADP bound/mol enzyme, indicating that the originally bound unlabeled ADP dissociates during the period of incubation and is replaced by the ADP in the solution. As further proof of the ability of bound ADP to exchange with the ADP in the bulk solution, the addition of a large excess of unlabeled ADP in a second step resulted in a drastic reduction in the radioactivity appearing in the column effluent, highlighting also the ability of the column to retain the radioactive ADP that is not bound to the enzyme.

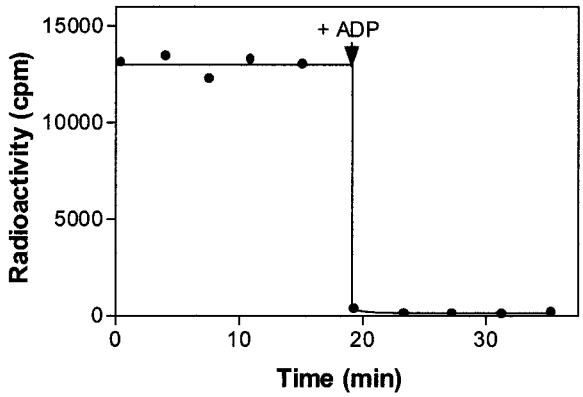

FIGURE 1: Time-course of enzyme labeling with [^{14}C]ADP and of exchange with unlabeled ADP. The enzyme (40 μM), in buffer A, was mixed with a trace of [^{14}C]ADP (185 $\times$ 10^3 cpm), and after the indicated time periods, 50 μL samples of the solution were subjected to centrifugal gel filtration. The arrow indicates the abrupt addition of 3.3 mM unlabeled ADP to the solution before additional samples were subjected to centrifugal gel filtration. The first of these samples was applied to the column 11 s after addition of the unlabeled ADP. Total radioactivity in the effluent of the columns was determined.

Exchange of enzyme-bound ADP with ADP in the solution appears complete after 0.5 min because centrifugal gel filtration performed after 28 s of incubation of the enzyme with the labeled ADP yields the same amount of the labeled enzyme that it does after longer periods of incubation (Figure 1). Furthermore, centrifugal gel filtration carried out only 11 s after the addition of an excess of unlabeled ADP to the

Table 2: ADP Sequestration by the Enzyme[a]

experiment	unlabeled ADP added	round of gel filtration	radioactivity in effluent (cpm/mg protein)
I	77 nmol	1	7125
	none	1	155 000
	none	4	170 000
	none	5	158 000
II	5 mM in the column	1	11 349
	none	1	242 178

[a] In experiment I, the enzyme (0.26 mg) in a volume of 0.13 mL was mixed with a trace of [^{14}C]ADP (56 000 cpm) and was split into two equal aliquots. Unlabeled ADP (77 nmol) was added to one of the aliquots, and 5 min later, 50 μL of each solution was subjected to centrifugal gel filtration. Samples from the effluents were taken for protein and radioactivity assay, and the remainder of the effluent from the sample that had not been supplemented with unlabeled ADP was applied 1 min after termination of the first centrifugal gel filtration to a second round of centrifugal gel filtration using a fresh column. Three subsequent rounds of gel filtration were carried out in the same way. In experiment II, a solution of 25 μM enzyme was incubated with a trace of [^{14}C]ADP (182×10^3 cpm/mL) in buffer A, and 50 μL samples were subjected to centrifugal gel filtration through columns equilibrated with buffer A alone or containing 5 mM ADP. Protein and radioactivity were assayed in samples taken from the effluents. In addition, the columns were extruded and sliced into four equal fractions, and the radioactivity in each slice was determined and is shown in Figure 2.

[^{14}C]ADP-labeled enzyme results in virtually complete exchange of the ADP bound to the enzyme, as shown by the decrease of the radioactivity in the column effluent (Figure 1, see results after the arrow), indicating that the exchange of bound ADP with the ADP in the solution has a $t_{1/2}$ value that is much shorter than 11 s. In fact, a small excess of radioactivity was found in the effluent of the sample centrifuged through the column 11 s after addition of the unlabeled ADP when compared to samples taken at longer periods (radioactivity at 11 s, 328 cpm; radioactivity at >11 s, 152 ± 16 cpm). If first-order kinetics for the exchange between bound and unbound ADP is assumed, then the comparison of the excess radioactivity at 11 s with the overall maximal decrease in the radioactive content of the effluent (12 880 cpm) yields a $t_{1/2}$ value of 1.8 s for the exchange.

Because enzyme-bound ADP exchanges relatively rapidly, we attempted to remove bound ADP by carrying out repeated cycles of centrifugal gel filtration because this technique is very effective in removing unbound compounds of low molecular weight (*19*). At least 1 min was allowed between successive gel filtrations to give ample time for dissociation of bound ADP. However, this approach failed to remove bound ADP: the same amount of labeled ADP relative to the amount of protein was present after the first, fourth, and fifth cycles of centrifugal gel filtration (Table 2). In contrast, when an excess of unlabeled ADP was added to the enzyme solution, the bulk of the radioactivity was removed in the first round of centrifugal gel filtration, demonstrating again the exchange of bound ADP with free ADP (Table 2). The lack of any substantial loss of ADP from the enzyme protein was confirmed by measurements of the radioactivity in the column (Figure 2). For this purpose, the column of gel was extruded and cut into four equal segments, as previously described (*20*). The small amount of radioactivity found in the column (8−10%) corresponded approximately to the amount of protein lost in each centrifugal gel filtration step and was evenly distributed throughout the segments, as would be expected if the label had remained associated with the lost protein. However, when excess unlabeled ADP was added to the enzyme prior to gel filtration (see above), not only was the majority of the labeled ADP retained by the column, but also the bulk of this radioactivity was present in the top and, to a lesser extent, in the second segment of the column, as would be expected for an unbound compound of low molecular weight (Table 2 and Figure 2).

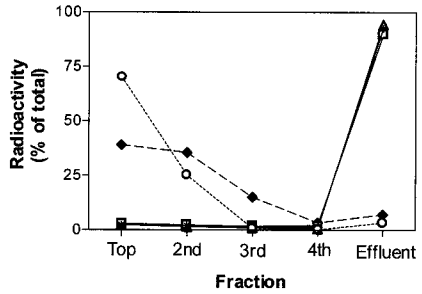

FIGURE 2: Radioactivity in sections of the column and in the effluent after centrifugal gel filtration of mixtures containing the enzyme and [^{14}C]ADP. The experiments are those of Table 2: Experiment I, (○) 77 nmol unlabeled ADP added to the mixture prior to gel filtration; (* and □) no unlabeled ADP added, fourth and fifth rounds of centrifugal gel filtration, respectively. Experiment II, (△) no unlabeled ADP added; (◆) 5 mM unlabeled ADP present in the column. The radioactivity is given as a fraction of the total radioactivity in the column and in the effluent.

Centrifugal gel filtration would be unable to remove bound ADP from the enzyme if ADP dissociation were too slow to take place during the centrifugal passage through the column. This possibility was excluded in experiments in which the column was equilibrated in buffer A containing 5 mM ADP. In these experiments, the majority of the radioactivity was retained in the column, as expected if bound [^{14}C]ADP had dissociated and had been replaced by the unlabeled ADP in the column (Table 2, experiment II). Nevertheless, the similarity of the radioactive content of the top and second column sections (Figure 2) and the substantial amount of radioactivity in the third segment indicate that ADP dissociation is not instantaneous but has a temporal frame of seconds because the time for the passage of the protein through the column is approximately 30 s (*19*).

Enzyme Binds ADP with Very High Affinity. The failure of centrifugal gel filtration to remove any substantial amount of bound ADP is compatible with the rapid exchange of the bound nucleotide only if the K_D value for the dissociation of ADP is extremely small relative to the amount of enzyme present. We could not easily remove bound ADP from the enzyme by dialysis. For example, after 80 h of dialysis versus 120 volumes of buffer, with 5 buffer changes, the enzyme retained approximately 50% of the [^{14}C]ADP bound initially. To estimate the K_D value for ADP, the enzyme was labeled with a trace of [^{14}C]ADP and was subjected to dialysis (see Materials and Methods). The fraction of ADP that was free in the dialysis bag was determined by comparison of the initial rate of outflow of the radioactivity from the bag (expressed as a fraction of the total radioactivity within the bag) after each buffer change with the corresponding rate when virtually all of the labeled ADP was set free by the addition of a large excess of unlabeled ADP to the dialysis bag. A Scatchard plot of the results (Figure 3) was linear

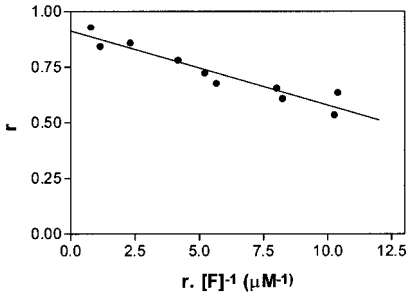

FIGURE 3: Scatchard plot for [^{14}C]ADP binding. The results of two rate of dialysis experiments (see Materials and Methods) are pooled. r = molar ratio of bound ADP to total enzyme. [F] = concentration of free ADP. The regression line yields a y-intercept of 0.91 ± 0.02 mol ADP/mol enzyme and a K_D value of 33.7 ± 3.7 nM.

Table 3: Trapping of Enzyme-Bound [^{14}C]ADP as [^{14}C]ATP in Pulse−Chase Experiments[a]

	chase step				^{14}C]ATP produced, percentage of total ^{14}C
experiment	[ADP], mM	interval, s	[carbamoyl-P], mM	chase period, s	
I	3.3	0	0.9	20	44.6
	3.3	60	0.9	20	4.1
	3.3	0[b]	0.9		2.4
II	6	0	1.7	30	71.6
	6	40	1.7	30	9.4
	3	0	1	40	79.7
	3	60	1	40	18.1
	3	0	10	40	84.4
	3	60	10	40	24.1
	3	0[b]	1		1.9

[a] A solution of the enzyme labeled with [^{14}C]ADP was mixed with small volumes of solutions of unlabeled ADP, after the indicated time interval to give carbamoyl phosphate (carbamoyl-P), to give the indicated concentrations of these reagents. A 0-s interval denotes the addition of ADP and carbamoyl phosphate as a mixture. At the indicated period after addition of the carbamoyl phosphate (chase period), 10% trichloroacetic acid was added (as a small volume of a stock 100% (w/v) solution), and the radioactivity in ATP was determined. In experiment I, 26 μM enzyme and 175 × 10^3 cpm/mL were used. In experiment II, the enzyme concentration was 180 μM, and the radioactivity was 327 × 10^3 cpm/mL. [b] The trichloroacetic acid was added before the unlabeled ADP and carbamoyl phosphate.

and was consistent with the existence of a single binding site for ADP per enzyme subunit with a K_D value of approximately 34 nM.

Pulse−Chase Experiments Trap Bound ADP as ATP Before It Dissociates. When the enzyme was incubated with [^{14}C]ADP and the reaction was "chased" by the rapid addition of carbamoyl phosphate and unlabeled ADP, a large excess of radioactivity was found in ATP compared with the radioactivity found when the unlabeled ADP was mixed with the enzyme 40−60 s before carbamoyl phosphate was added (Table 3). Thus, under these conditions, bound ADP can react with carbamoyl phosphate to yield ATP before it dissociates from the enzyme. The efficiency of trapping bound ADP is quite high (Table 3), and it does not increase markedly by increasing the concentration of carbamoyl phosphate from 1 to 10 mM. However, the radioactivity in ATP is higher after 30−40 s of reaction with carbamoyl phosphate than when the chase period lasts only 20 s. This aspect of the chase reaction is examined in more detail later.

Determination of the Rate of Exchange of Bound ADP with ADP in Solution Using Pulse−Chase Studies and Demonstration That the Binding of ADP to the Enzyme Is Very Rapid. Figure 4 illustrates the influence of variable time intervals between the additions of unlabeled ADP and carbamoyl phosphate on the radioactivity incorporated into ATP in pulse−chase experiments (Figure 4). The radioactivity in ATP declines sharply with longer time intervals, and the data fits well to an exponential with a $t_{1/2}$ value of 2.43 s. Because the experiments were carried out under conditions where the enzyme was essentially saturated with ADP, $t_{1/2}$ is a measure of the rate constant for dissociation of this nucleotide, k_2. From the $t_{1/2}$ value, k_2 was determined to be 0.285 s^{-1}. The $t_{1/2}$ value for ADP dissociation determined in this way is consistent with the rough estimate provided above on the basis of the centrifugal gel filtration data (Figure 1).

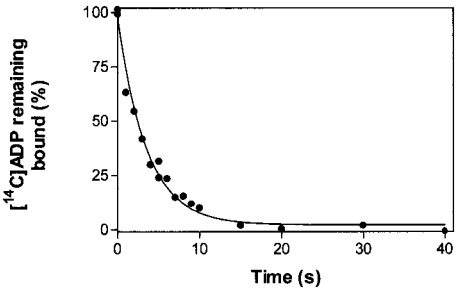

FIGURE 4: Time course of ADP dissociation from the enzyme, monitored by pulse−chase. A portion (0.5 mL) of a 0.85 mg/mL solution of the enzyme in buffer A was mixed with a trace of [^{14}C]-ADP (235 × 10^3 cpm/mL) and was magnetically stirred in a 2 mL beaker. Then 0.05 mL of 36.5 mM unlabeled ADP was rapidly stirred in, followed, after the indicated time interval, by 0.05 mL of 30 mM carbamoyl phosphate. Time 0 corresponds to the addition of ADP and the carbamoyl phosphate as a mixture in 0.1 mL. Forty seconds after the addition of the carbamoyl phosphate, 0.025 mL of 100% (w/v) trichloroacetic acid was added, and the radioactivity in ATP was determined (see Materials and Methods). The results have been corrected for the presence of 1.9% [^{14}C]ATP in [^{14}C]-ADP (determined by addition of the trichloroacetic acid before mixing in the unlabeled ADP and carbamoyl phosphate) and for the production of ATP of low specific activity during the 40 s incubation with carbamoyl phosphate (determined in samples in which the carbamoyl phosphate was added 60 s after the ADP; for the correction, it was taken into consideration that a smaller amount of radioactivity exchanges if bound ADP gives ATP before dissociation). Results are expressed as a percent fraction of the radioactivity found in ATP when carbamoyl phosphate and ADP were added together (74% of the radioactivity in the sample). The line fitted to the points is the exponential for a decay with a $t_{1/2}$ value of 2.43 s.

The rate constant for the binding of ADP to the enzyme, k_1, may be calculated from the relationship $K_D = k_2/k_1$, where K_D is the dissociation constant for ADP and has the value determined above of 33.7 × 10^{-9} M. This calculation gives a second-order rate constant for ADP binding of approximately 8.4 × 10^6 s^{-1} M^{-1}, a very large value that is only about 2 orders of magnitude lower than might be expected for a diffusion-controlled process (*25*). For an ADP concentration of 1 mM, the rate of binding would be 8.4 × 10^3 s^{-1}, a value to be compared with a turnover rate for the enzyme of about 0.06 s^{-1} (discussed later).

Conversion of Bound ADP to ATP Limits the Rate of ATP Synthesis. In the pulse−chase experiments described in Table 3 with a chase period of 40 s, conversion of bound ADP to ATP was on the order of 75%[2], but with a 20-s chase period, the yield of ATP was substantially less. Further studies showed ATP to be formed in the chase period with an

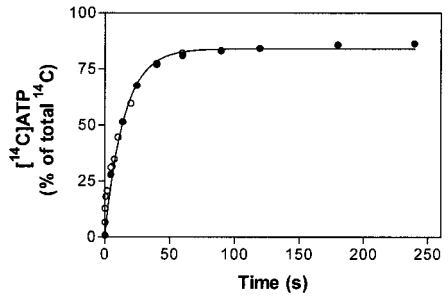

FIGURE 5: Time course of the conversion of bound [^{14}C]ADP to [^{14}C]ATP upon reaction with carbamoyl phosphate. A solution of 1 mg/mL enzyme in buffer A was incubated with a trace of [^{14}C]-ADP (213×10^3 cpm/mL). A 0.5 mL sample of this solution was placed in a 2 mL beaker, and 50 μL of 30 mM carbamoyl phosphate, alone (open symbols) or with 26.5 mM ADP (closed symbols), was added abruptly with brisk stirring. After the indicated time periods, 20 μL of the solution was mixed with 2 μL of 100% (w/v) trichloroacetic acid. Alternatively, for short intervals between the addition of the carbamoyl phosphate and the quenching with acid, 25 μL of 100% trichloroacetic acid was added abruptly to the solution in the beaker at the indicated time, after the addition of carbamoyl phosphate. The line drawn corresponds to the exponential for 84% maximal conversion and a $t_{1/2}$ value of 10.3 s.

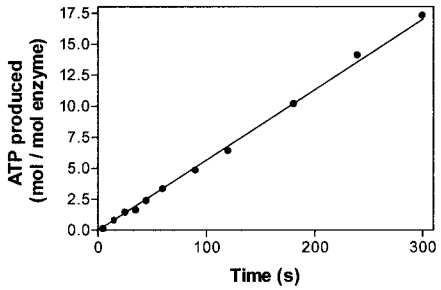

FIGURE 6: Time-course of ATP production by the enzyme. A portion (0.5 mL) of a solution of the enzyme (0.94 mg/mL) in buffer A was mixed with a trace of [^{14}C]ADP (201×10^3 cpm/mL) and with 46 μL of 30 mM unlabeled ADP, and the mixture was incubated 2 min. Then, 50 μL of 30 mM carbamoyl phosphate was suddenly stirred into the solution, and samples were quenched with 5% trichloroacetic acid at timed intervals after the addition of the carbamoyl phosphate, and the amount of [^{14}C]ATP in the quenched samples was determined. The results are corrected for the amount of [^{14}C]ATP in samples that were quenched with acid before the addition of carbamoyl phosphate. The regression line ($r = 0.998$) is extrapolated through the origin and gives a rate of ATP production of 0.057 s^{-1}.

exponential time course and a $t_{1/2}$ value of 10.3 s (Figure 5, closed circles). Chase experiments were also carried out without the addition of unlabeled ADP in the chase because in the pulse period with labeled ADP only a very small fraction of the ADP remains unbound, as is discussed above. The data from these experiments (Figure 5, open circles) agree closely with those obtained with excess unlabeled ADP in the chase (Figure 5, closed circles).

The above findings are inconsistent with a simple model in which ADP dissociates at the same rate from both the enzyme−ADP complex ($t_{1/2}$ for dissociation, 2.43 s) and from the ternary enzyme−ADP−CP complex that is formed in the chase. If ADP dissociated from both complexes at the same rate, to convert in the chase period 75% of bound ADP into ATP would require this conversion to be 3-fold faster than that of the dissociation of bound ADP, for which the $t_{1/2}$ value is 2.43 s, leading to an expected $t_{1/2} < 1$ s, whereas the experimentally determined $t_{1/2}$ value for the conversion of bound ADP to ATP was 10.3 s (Figure 5). The high degree of conversion of bound, labeled ADP to ATP and the similar efficiency of the conversion irrespective of the presence or absence of unlabeled ADP in the chase solution (Figure 5) are explained best if, initially, in the chase, carbamoyl phosphate binds quickly (compared with the speed of ADP dissociation) to form the ternary enzyme−ADP−CP complex, from which ADP cannot dissociate or dissociates only very slowly. Thus, almost immediately after the addition of the chase, there will be only a single precursor (enzyme−ADP−CP) for ATP generation in the chase solution, which also provides an explanation for the exponential shape of the curve depicting the production of ATP in the chase. With two precursors in series (enzyme−ADP and enzyme−ADP−CP), a double exponential curve would otherwise have been expected, with an initial "lag" in the production of ATP when compared with that from a simple exponential.

In the scheme shown in Figure 7, the conversion of ADP into ATP and the dissociation of ATP from the enzyme are shown as separate steps. The effect of the dissociation step on the kinetics depends on the relative values of the rate constants. To further investigate this aspect of the reaction, experiments were carried out under the same conditions as were the pulse−chase experiments, but the enzyme was equilibrated with an excess of ADP and then the reaction was simply started by the addition of carbamoyl phosphate (Figure 6). The rate of the reaction was constant, and the turnover rate (0.057 s^{-1}) was only slightly less than the rate of production of ATP from the enzyme−ADP−CP complex found in the pulse−chase experiments (0.068 s^{-1}). Therefore, although slow, the conversion of enzyme-bound ADP to ATP monitored in Figure 5 is sufficiently fast to be a part of the normal enzyme reaction, and given the similarity of its rate and of the turnover rate, this step is likely to be the main rate-limiting process of the reaction. Consequently, virtually all of the enzyme, under the steady-state conditions, must be present as the enzyme−ADP−CP complex; correspondingly, the amount of the enzyme in ATP-containing or other complexes must be small. Further, the progress of the reaction is linear with respect to time and passes through the origin (Figure 6). If there were a significant slow step after the formation of ATP, then initially there would be a rapid phase in the reaction while the intermediates containing ATP increased to a sufficient concentration to maintain the reaction at its steady-state rate, which would lead to a positive intercept at zero time. Considering the possibility of a slow dissociation of ATP from the enzyme−ATP−carbamate complex (Figure 7), it can be shown that the steady-state rate of ATP production (mol/s mol enzyme) approximates to $k_5k_7/(k_5 + k_6 + k_7)$ and that the intercept (mol/mol enzyme) approximates to $k_5/(k_5 + k_6 + k_7)$. The ratio of these values yields k_7. From Figure 6, it would appear that if there is an intercept, it is very unlikely to exceed 0.2 mol/mol enzyme. Using this value and the steady-state rate of 0.057 s^{-1}, $k_7 \geq 0.057/0.2 = 0.285$ s^{-1}, corresponding to a $t_{1/2}$ value of 2.4 s

[2] Estimated from Table 3, by subtracting from the results of the 0-s interval tubes the radioactivity found after a 60-s interval, adjusted to take into account the lower specific activity of the ADP in free solution in the tubes with the 0-s interval, where little labeled ADP dissociates from the enzyme.

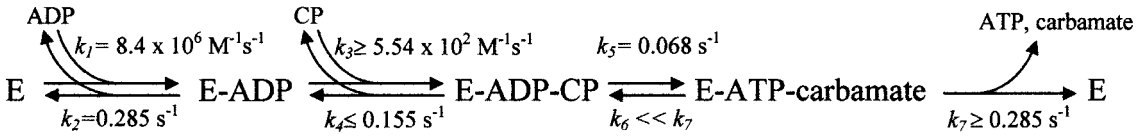

FIGURE 7: Steps and values for the rate constants in the reaction of the carbamate kinase from *P. furiosus* at 24 °C (E, enzyme; CP, carbamoyl phosphate). k_2 was determined from the time-course of ADP dissociation and k_1, from the expression $K_D = 34$ nM$= k_2/k_1$. The minimum value given for k_3 was estimated from the ratio $k_3 \times 2.7 \times 10^{-3}$ M $\geq k_2 \times 0.84/0.16$, which is based on 84% trapping of bound ADP as ATP in pulse−chase experiments in the presence of 2.7 mM carbamoyl phosphate. k_4 is assumed not to exceed $0.1 \times k_3 \times 2.7 \times 10^{-3}$ M because 2.7 mM appears to stop the dissociation of ADP completely; thus, at this concentration of carbamoyl phosphate, the equilibrium between the binary and ternary complexes must greatly favor the ternary complex, from which ADP cannot dissociate. k_5 has been given the value of the rate constant for the conversion of enzyme-bound ADP to ATP, illustrated in Figure 5, which is based on the exponential shape of the time-course of the conversion and on the lack of an intercept in the steady-state reaction, indicating that the concentration of the enzyme−ATP−carbamate complex is negligible. The estimation of a minimal value for k_7 is given at the end of the Results section. k_6 has to be small compared to k_7 because in the steady-state assays the slope $= 0.057$ s$^{-1} = k_5 \times k_7/(k_7 + k_5 + k_6)$. Taking the value of k_5 as 0.068 s^{-1}, $k_7/(k_7 + k_5 + k_6) = 0.84$, thus $(k_5 + k_6)/k_7 = (1-0.84)/0.84 = 0.19$.

for product dissociation, which is much faster than the conversion of bound ADP into ATP ($t_{1/2} = 10.3$ s). Thus, both the rates of individual steps and the lack of a significant intercept support the conclusions that no substantial amounts of E−ATP complexes were present in the steady-state experiment illustrated in Figure 6 and that the ATP measured in the pulse−chase experiments of Figure 5 represents ATP that has dissociated from the enzyme.

DISCUSSION

The present demonstration that enzyme-bound ADP exchanges with ADP in the solution but that the affinity for ADP binding is extremely high (34 nM) proves our previous proposal that ADP might be unable to dissociate from the enzyme (*14*) incorrect and fits very well with our observations on the 3-D structure of the enzyme−ADP complex that revealed the existence of extensive interactions and tight fitting between the protein and the nucleotide (Figure 8) but that failed to substantiate a closure mechanism that would lock the ADP into its binding site (*14*). The nucleotide was shown to bind in a tightly fitting crevice, with the adenine occupying a narrow pocket (Figure 8) that was sandwiched between a tyrosine and a methionine, the ribose half-buried on a ridge with its hydroxyl oxygens partially exposed to the medium, and the two phosphates nested at the protein surface, interacting with the protein only through contacts of the β phosphate mainly with two lysines. Structural differences at the site for ADP between the carbamate kinase from *P. furiosus* and the mesophilic enzyme from *E. faecalis* (*14*, *15*) appear to be in line with the observation of a much lower affinity of the enterococcal enzyme for ADP (*26*): the site for ADP is more open in the mesophilic enzyme from *E. faecalis*, and some interactions between the enzyme and the nucleotide may be missing (*14*).

The low K_D value rather than restrictions to ADP dissociation accounts for the presence of bound ADP in the protein crystals and for the inefficiency of the techniques used for the removal of ADP from the hyperthermophilic enzyme. Thus, for 5 mg/mL enzyme in the mother liquor and an ADP content of 0.93 mol/mol enzyme, free [ADP] should be 0.43 μM, which is more than 10 times larger than the K_D value. Therefore, 99.7% of the ADP in the crystallization mixture will be bound to the enzyme. In the preparation of the enzyme solution for crystallization, the procedure used for buffer exchange, consisting of several cycles of dilution (to 0.5 mg/mL) and concentration (to 10 mg/mL) by ultrafiltration, should remove only ≤2.3% of the ADP per cycle if the enzyme initially contains 0.93 mol ADP/mol enzyme and the K_D value is 34 nM. Centrifugal gel filtration also proved of little use to remove bound ADP, as shown by the retention of the enzyme-bound ADP even

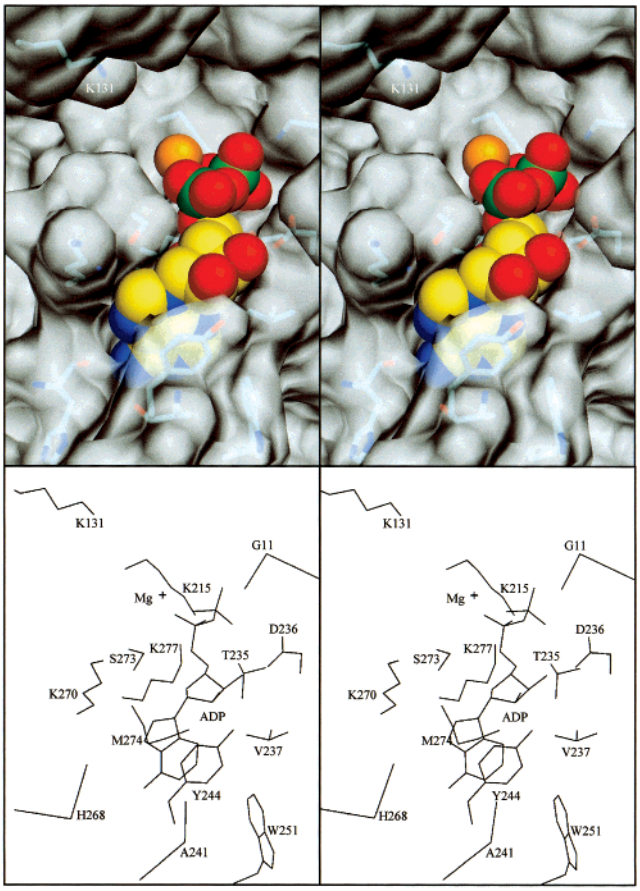

FIGURE 8: Structure of the ADP site of *P. furiosus* carbamate kinase. Stereopairs were prepared using the coordinates deposited in RCSB Protein Data Bank 1e19. The top panels provide a semitransparent surface representation of the site, with a space-filling model of bound MgADP to highlight the tight fit of the ligand to the site. Color code for ADP atoms is yellow for carbon, blue for nitrogen, red for oxygen, green for phosphorus, and orange for magnesium. The backbones of the residues that form the site are presented under the surface. Only Lys131 is labeled (constructed with Program DINO (*27*)). The bottom panels identify in bond representation the binding site residues and the bound ADP molecule, with the Mg shown as a cross. G11, H268, and A241 correspond to the αC of these residues, shown as a part of the αC backbones (drawn with MOLSCRIPT (*28*)).

after five successive rounds of centrifugal gel filtration (see Results), perhaps because the noninstantaneous dissociation of ADP from the enzyme drastically limits the number of theoretical plates of the chromatographic process. Finally, in the case of dialysis, for 30 μM enzyme containing 0.93 bound mol ADP/mol enzyme, free [ADP] = 0.39 μM for a K_D value of 34 nM. By assuming that in 1 h 50% of the free ADP exits the dialysis bag (on the basis of our observations when all of the ADP was free), 0.2 nmol ADP would exit per hour. If this rate remained constant (clearly an overestimation), approximately 70 h would be required for removal of 50% of bound ADP, in line with our observation of 80 h of dialysis for approximately 50% removal.

The same reasons must underlie the observation of bound ADP in the enzyme preparation, even when no nucleotide was added during the purification (*14*). The ADP must have been bound to the enzyme in vivo or in the initial cell extract and must have been carried in its bound form throughout the entire purification process. As already reported (*13*), approximately 10% of the cell protein was the plasmid-encoded, superexpressed pyrococcal enzyme, corresponding to an estimated concentration in the cells of 20 mg/mL or 0.5 mM enzyme. Because the dilution factors in the three-step purification process (*10*) did not exceed 50 and the two dialysis steps used were each <6 h and did not involve more than three changes of the dialysate, final occupancy of the site for ADP was ≥0.8.

Under the conditions in our experiments where the amount of ADP was limited (Figure 5), the opportunity was taken to test for possible interactions between the two active centers of the carbamate kinase dimer. Given the limited ADP availability, when ATP is formed at one active center, no ADP binds to the enzyme to replace the ATP that has been formed. Thus, conversion of ADP to ATP at the second site occurs under different conditions, and if there were cross-talk between the two active centers of the dimer, the formation of ATP under these conditions might have been expected to follow a time course represented by a double exponential. However, as already shown in Figure 5, ATP formation followed exactly the same exponential time course whether or not additional ADP was added to ensure the presence of an ADP at both binding sites, indicating that there is no functional cross-talk between the two sites of the dimer.

The present work provides information about the reaction pathway and the rate constants of the various steps (Figure 7). The rate of addition of carbamoyl phosphate must be high to account for the high degree of conversion of ADP to ATP in pulse−chase experiments (Figure 5), allowing us to estimate a minimum value for this rate constant ($k_3 \geq 5.54 \times 10^2$ M^{-1} s^{-1}). At millimolar carbamoyl phosphate concentrations, this step is much faster than the turnover of the enzyme in the direction of ATP production. As already indicated, the high efficiency of trapping ADP also indicates that when carbamoyl phosphate binds the dissociation of ADP is effectively stopped, which constitutes strong evidence for a compulsory order of addition of ADP and carbamoyl phosphate because if ADP dissociates only slowly from the ternary complex the rate constants for the alternative reaction in which carbamoyl phosphate adds first to the enzyme must be very unfavorable. That ADP binds first is consistent with the isolation of the E−ADP complex and was originally proposed for mesophilic carbamate kinase on the basis of the results of kinetic studies under steady-state conditions (*26*). The dissociation of carbamate and ATP may be assumed to be ordered in view of the evidence for the binding of ADP before that of carbamoyl phosphate, our preliminary finding that ATP displaces enzyme-bound ADP in absence of other substrates (unpublished results), and the conclusions derived from steady-state kinetic studies with the carbamate kinase from *E. faecalis* (*26*). The failure to find a positive intercept in studies on the steady-state reaction (Figure 6) also indicates that the dissociation of carbamate and ATP must be rapid in relation to the rate of ATP formation from bound ADP and carbamoyl phosphate, allowing one to propose a minimum value for the rate of product release, $k_7 \geq 0.285$ s^{-1}. Overall, our study identifies the isomerization between enzyme-bound substrates and products as the slowest step in the reaction pathway at 24 °C and excludes the dissociation of ATP, which is much faster, that limits the rate of the reaction. Given the similarity of the rates of the forward and reverse reactions catalyzed by this enzyme (at least at 37 °C, (*13*)), it also appears unlikely that the rate of dissociation of ADP, which takes place with a $t_{1/2}$ value of 2.43 s, limits the rate of the reverse reaction, the synthesis of carbamoyl phosphate from ATP and carbamate. Therefore, hyperthermophilic carbamate kinase does not resemble thermophilic indoleglycerol phosphate synthetase (*4*). In the latter enzyme, product dissociation limited the rate at mesophilic temperatures, whereas in the carbamate kinase the isomerization between two central complexes is the limiting step at low temperature, showing that diverse causative mechanisms in different thermophilic enzymes may account for their sluggishness at low temperature.

The slow isomerization may indicate that the chemical process of phosphoryl group transfer is the step that is slowed the most at low temperature in the carbamate kinase from *P. furiosus*. As catalysis exquisitely depends on the proper orientation of groups at specific locations (*25*) and because enzymes are known to fluctuate between many different conformations, as highlighted by the results of NMR studies, the frequency of adoption of a specific enzyme conformation may determine the rate of the catalytic process. At low temperature, the catalytically active conformation would be adopted by this enzyme very infrequently; therefore, it is likely that the snapshot provided by the crystallographic structure of the enzyme corresponds to a catalytically inactive form. In any case, further structural information is required to clarify the nature of the rapid change that is triggered by the binding of carbamoyl phosphate and that results in the sequestration of enzyme-bound ADP.

Movement of Lys131 toward the active center appears to be required for efficient catalysis of phosphoryl group transfer by this enzyme. This residue, which is fully conserved in carbamate kinases (*12*) and whose mutation to alanine inactivates the enzyme (unpublished results of this laboratory with the carbamate kinase from *E. faecalis*), was proposed (*14*) to be important for negative charge neutralization in the transition state. Lys131 emerges from the so-called "flexible subdomain" (colored in yellow in Figure 9) in the direction of the active center, but it is too far away to interact with the substrates (*14*, *15*) (Figures 8 and 9), thus the "flexible subdomain" has to move toward the active center to allow the participation of the lysine in the catalysis

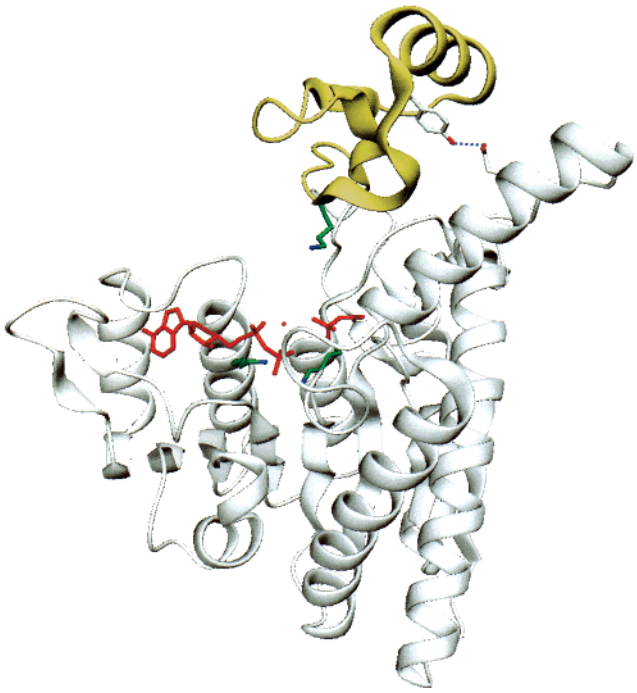

FIGURE 9: Ribbon representation of the *P. furiosus* carbamate kinase subunit. MgADP and a modeled (*14*) bound carbamoyl phosphate are shown in red. The side chains of lysines 131, 215, and 277 are shown in green, with the ϵ-amino group in blue. Lys131 emerges from the flexible subdomain (in yellow). Tyr137 is also shown emerging from the flexible subdomain, forming a hydrogen bond with aspartate 65 from helix B (drawn with DINO (*27*)).

of the reaction. The ability of this subdomain to move may be reflected in the large temperature factors (RCSB Protein Data Bank accession numbers 1b7b and 1e19) and important differences of the X-ray structures of the enterococcal and pyrococcal enzymes for this subdomain (*14*). Among the differences, a hydrogen bond between Tyr137 and Asp65 that links the flexible subdomain to α helix B is present in pyrococcal but not in enterococcal carbamate kinase (illustrated in Figure 9) and may render the movement of the subdomain toward the active center in the pyrococcal enzyme more difficult. Given the location of the proposed (*14*) site for carbamoyl phosphate (Figure 9), it is conceivable that the binding of this substrate may trigger the approach of the subdomain, completing the closure of the active site and resulting in the sequestration of bound ADP in the ternary complex that was demonstrated in the present experiments. However, given the slow phosphoryl group transfer within this ternary complex, additional movements over shorter ranges may be needed for proper positioning of Lys131 for catalysis.

REFERENCES

1. Jaenicke, R., and Böhm, G. (1998) *Curr. Opin. Struct. Biol. 8*, 738−748.
2. Ladenstein, R., and Antranikian, G. (1998) *Adv. Biochem. Eng./Biotechnol. 61*, 37−85.
3. Varley, P. G., and Pain, R. H. (1991) *J. Mol. Biol. 220*, 531−538.
4. Merz, A., Yee, M., Szadkowski, H., Pappenberger, G., Crameri, A., Stemmer, W. P. C., Yanofsy, C., and Kirschner, K. (2000) *Biochemistry 39*, 880−889.
5. Yip, K. S., Stillman, T. J., Britton, K. L., Atymiuk, P. J., Baker, P. J., Sedelnikova, S. E., Engel, P. C., Paquo, A., Chiaraluce, R., Consalvi, V., Scandurra, R., and Rice, D. W. (1995) *Structure 3*, 1147−1158.
6. Korolev, S., Naval, M., Barnes, W. M., Di Cera, E., and Waksman, G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9264−9268.
7. Villeret, V., Clantin, B., Tricot, C., Legrain, C., Roovers, M., Stalon, V., Glansdorff, N., and Van Beeumen, J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 2801−2806.
8. Aguilar, C. F., Sanderson, I., Moracci, M., Ciaramella, M., Nucci, R., Rossi, M., and Pearl, L. H. (1997) *J. Mol. Biol. 271*, 789−802.
9. Kohen, A., Cannio, R., Bartolucci, S., and Klinman, J. P. (1999) *Nature 399*, 496−499.
10. Uriarte, M., Marina, A., Ramón-Maiques, S., Rubio, V., Durbecq, V., Legrain, C., and Glansdorff, N. (2001) *Methods Enzymol. 331*, 236−247.
11. Durbecq, V., Legrain, C., Roovers, M., Pierard, A., and Glansdorff, N. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12803−12808.
12. Marina, A., Uriarte, M., Barcelona, B., Fresquet, V., Cervera, J., and Rubio, V. (1998). *Eur. J. Biochem. 253*, 280−291.
13. Uriarte, M., Marina, A., Ramón-Maiques, S., Fita, I., and Rubio, V. (1999) *J. Biol. Chem. 274*, 16295−16303.
14. Ramón-Maiques, S., Marina, A., Uriarte, M., Fita, I., and Rubio, V. (2000) *J. Mol. Biol. 299*, 463−476.
15. Marina, A., Alzari, P. M., Bravo, J., Uriarte, M., Barcelona, B., Fita, I., and Rubio, V. (1999) *Protein Sci. 8*, 934−940.
16. Bradford, M. M. (1976). *Anal. Biochem. 72*, 248−254.
17. Dawson, R. M. C., Elliot, D. P., Elliot, W. H., and Jones, K. M. (1986) *Data for Biochemical Research* 3rd ed., p 105, Oxford University Press, Oxford.
18. Bergmeyer, H. U. (1986) *Methods of Enzymatic Analysis* 3rd ed., Vol. 7, pp 634−635, VCH Verlagsgesellschaft, Weinheim, Germany.
19. Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
20. Britton, H. G., and Rubio, V. (1988) *Eur. J. Biochem. 171*, 615−622.
21. Colowick, S. P., and Womack, F. C.(1969) *J. Biol. Chem. 244*, 774−777.
22. Randerath, K., and Randerath, E. (1964) *J. Chromatogr. 16*, 111−125.
23. Rubio, V., Britton, H. G., Grisolia, S., Sproat, B. S., and Lowe, G. (1981) *Biochemistry 20*, 1969−1974.
24. Powers, S. G., and Meister, A. (1978) *J. Biol. Chem. 253*, 1258−1265.
25. Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.
26. Marshall, M., and Cohen, P. P. (1966) *J. Biol. Chem. 241*, 4197−4208.
27. Philippsen, A. (1998) *Dino, a Visualization System for Structural Data*, http://www.bioz.unibas.ch/~xray/dino.
28. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

BI011637D