Heterodimeric Deoxynucleoside Kinases of *Lactobacillus acidophilus* R-26: Functional Assignment of Subunits Using Limited Proteolysis Controlled by End-Product Inhibitors†

Seiichiro Ikeda, Grace T. Ma,‡ and David H. Ives*

Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210

Received January 7, 1994; Revised Manuscript Received February 25, 1994®

ABSTRACT: Heterodimeric quaternary structures for two enzyme complexes from *Lactobacillus acidophilus* R-26 exhibiting deoxycytidine kinase/deoxyadenosine kinase(I) and deoxyguanosine kinase/deoxyadenosine kinase(II) activities have been proven by the following steps: (1) separation of each complex into two components on SDS–PAGE at pH 6.6; (2) N-terminal amino acid sequencing of each component; (3) functional assignment of each component by differential limited proteolysis. The third step was facilitated by the finding that the binding of a specific end-product inhibitor, dNTP, to each kinase active site makes the corresponding kinase subunit resistant to trypsin, while leaving the heterologous kinase subunit susceptible to proteolysis. Analysis on SDS–PAGE has revealed only two fragments (15.8 and 11.0 kDa) following proteolysis of dCyd kinase/dAdo kinase(I) with trypsin in the presence of dATP. This may indicate that the kinase polypeptide chain (27.2 kDa) not protected by dNTP is cut by trypsin at a single specific site, with concomitant loss of activity. Thus, this work presents a unique approach to the clarification of structure and function of enzymes composed of heterologous subunits.

Lactobacillus acidophilus R-26 is one of the few prokaryotes capable of using all four deoxynucleosides as precursors of DNA. Lacking a functional ribonucleotide reductase, this strain is therefore dependent on four deoxynucleoside kinase activities. In addition to a thymidine kinase which closely resembles that found in other bacteria, the other three activites are organized into two paired deoxynucleoside kinase complexes, namely, kinase complex I [dCyd[1] kinase/dAdo kinase-(I), dCK/dAK] and kinase complex II [dGuo kinase/dAdo kinase(II), dGK/dAK] (Deibel & Ives, 1977a,b; Ikeda & Ives, 1985). Several remarkable features distinguish these kinase complexes from the deoxynucleoside kinases previously found in either prokaryotes or eukaryotes. Additive activities and the absence of mutual competition indicated that the paired kinase activities are located at two separate sites—possibly on separate subunits—and have nearly absolute specificities for their respective nucleoside substrates, in contrast to the broadly-specific mammalian deoxycytidine kinase. Each of these enzymes thus catalyzes the first committed steps of parallel biosynthetic pathways. In addition, positive allosteric interactions between sites (or subunits) appear to contribute to the production of balanced quantities of nucleotides, despite the duplication of dAdo kinase (Deibel et al., 1977; Chakravarty et al., 1984). Finally, each activity is strongly inhibited by its homologous deoxynucleoside triphosphate end-product (dNTP)—but apparently not at an allosteric binding site. The kinetic effects of each dNTP are identical to those of the respective multisubstrate inhibitor (Deibel & Ives, 1977a; Ikeda et al., 1986). Therefore, it has been proposed that the binding site for a dNTP is the active site; the simplest explanation consistent with all the evidence is that the deoxynucleoside moiety of dNTP fits into the deoxynucleoside binding site, while the triphosphate portion of dNTP interacts with the triphosphate binding site for ATP in a "reversed" orientation relative to that of ATP (Ikeda et al., 1986).

Kinase complexes I and II were both purified to homogeneity by affinity chromatography on dCTP–Sepharose (Ikeda et al., 1988) or dATP–Sepharose (Ikeda et al., unpublished results). With a native mass of 50–56 kDa, each enzyme appeared to be composed of two subunits of similar size (27.2 kDa), but it had yet to be determined whether the two subunits in each complex comprise a homodimer, each carrying two catalytic sites on each polypeptide chain, or if each protein is a heterodimer carrying distinct functions on separate subunits. We present evidence for the latter in this paper.

There are many examples in which a single ligand, such as a coenzyme, substrate, or inhibitor, can drastically affect the rate of proteolysis of the entire enzyme molecule (Mihalyi, 1972). For example, hexokinase from yeast shows complete resistance to trypsin when 1 mol of glucose is bound to the enzyme, reflecting a substantial conformational change at the active-site cleft upon substrate binding (Trayser & Colowick, 1961). Simiarly, the binding of specific dNTPs to the active sites of deoxynucleoside kinases from *Lactobacillus* might also selectively protect the homologous subunits from proteolysis.

EXPERIMENTAL PROCEDURES

Materials. dNTP, TPCK-treated trypsin from bovine pancreases, and trypsin inhibitor from soybean were obtained from Sigma Chemical Co. Tritiated deoxynucleosides were from ICN. Reagents for gel electrophoresis were supplied by

† This work was supported by Grants 5R01CA-47828 and 2R01GM-49635 from the National Institutes of Health.

* To whom correspondence should be addressed. Telephone: (614)-292-0485. FAX: (614)292-6773.

‡ Present address: Department of Neurobiology & Physiology, Northwestern University, Evanston, IL 60208-3520.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: dCyd, deoxycytidine; dAdo, deoxyadenosine; dGuo, deoxyguanosine; dNTP, deoxynucleoside triphosphate; dCK, dCyd kinase; dAK, dAdo kinase; dGK, dGuo kinase; dCp_4–Sepharose (dCTP–Sepharose), dCyd 5′-tetraphosphate-bound Sepharose; dAp_4–Sepharose (dATP–Sepharose), dAdo 5′-tetraphosphate-bound Sepharose; dAp_4A–Sepharose, deoxyadenosine 5′-adenosine 5‴-P^1,P^4-tetraphosphate-bound Sepharose; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Bio-Rad. Immobilon-P PVDF membrane was obtained from Millipore.

Enzyme Preparations and Assays. A homogeneous preparation of kinase complex I [dCyd kinase/dAdo kinase(I)] from *Lactobacillus acidophilus* R-26 was obtained by affinity chromatography on dCTP–Sepharose as described previously (Ikeda et al., 1988). Kinase complex II [dGuo kinase/dAdo kinase(II)] was purified to homogeneity by applying the run-through fraction from dCTP–Sepharose to a newly constructed dATP–Sepharose column, eluting with dATP (Ikeda et al., unpublished data). At the final step of purification, kinase complex I or II was eluted from the affinity column with the eluent, 0.5 mM dCTP (or dATP), and was concentrated to a minimum volume using a Centricon-10 concentrator (Amicon). The preparations were stored at −20 °C in the presence of the eluent dNTP for best stability. For the experiments involving differential proteolysis, the 0.5 mM dNTP remaining in the enzyme preparation was reduced to less than 0.1 μM by repeated dilution with buffer A (15 mM potassium phosphate buffer, pH 8.0, containing 20% glycerol) and reconcentrated using a Centricon concentrator. Then, an appropriate dNTP (0.1 mM) was added to the enzyme solution in order to protect a specific subunit from proteolysis. dCyd kinase, dAdo kinase, and dGuo kinase assays were carried out radiometrically as described previously (Deibel & Ives, 1977b; Ives, 1984). The final concentration of each component was as follows: 20 μM deoxynucleoside, 0.5 μCi of [^{3}H]deoxynucleoside, 10 mM ATP, and 12 mM $MgCl_2$ in 40 μL of 0.1 M Tris-HCl, pH 8.0. One unit of activity is defined as the amount producing 1 nmol of deoxynucleoside monophosphate per minute at 20 °C. Protein determinations employed the Bradford method (Bradford, 1978).

Gel Electrophoresis. The discontinuous Tris–glycine/Tris-HCl buffer system (Laemmli, 1970) was used for both nondenaturing PAGE (8–15% acrylamide) and SDS–PAGE (12 or 15% acrylamide), with a Bio-Rad Model 360 mini vertical slab-cell (83-mm-long gel plate). For the separation of subunits on SDS–PAGE, the MZE 3328.IV buffer system (Moos et al., 1988) was used. A step-gradient of acrylamide (12.0/13.2%) in a 121-mm-long gel slab gave even better resolution. Electrophoresis was stopped as soon as the bands for both subunits entered the 13.2% acrylamide portion of the gel. Protein bands were visualized by staining with Coomassie Blue R-250. The relative intensity of each stained band was quantitated using a scanning densitometer (Hoefer Scientific Instruments). For N-terminal amino acid sequencing of separated subunits, the separating gel was cast 1 day before use and was subjected to preelectrophoresis in separating gel buffer containing 0.1 mM thioglycolate (Moos et al., 1988). In order to locate the kinase activity peak on nondenaturing gels, one channel of the gel was cut into 2-mm slices and assayed as described previously (Deibel & Ives, 1977a).

Electrophoretic Transfer. A semidry protein-transfer system (Kyhse-Andersen, 1984) was used to transfer subunit proteins separated on SDS–PAGE to a PVDF membrane. Protein bands were visualized on the membrane by staining with 0.5% Coomassie Blue R-250 in 40% methanol for 5 min and destaining with 50% methanol (Speicher, 1989). The stained subunit bands were cut out with a scalpel and submitted for N-terminal protein sequencing analysis by automated gas-phase Edman degradation chemistry on an Applied Biosystems Model 470A sequencer at The Ohio State University Biochemical Instrument Center.

Differential Limited Proteolysis of Kinase Complexes with Trypsin. Purified kinase complex I or II (1.0 μg in 20 μL of buffer A) was incubated at 20 °C with trypsin (2–10% w/w) for up to 30 min. To protect a specific subunit from proteolysis, the corresponding dNTP (0.1 mM) was included in the digestion mixture. To stop the proteolysis, trypsin inhibitor (2 molar ratio over trypsin) or PMSF (final concentration 1.0 mM) was added to the reaction mixture. This reaction mixture was applied directly to nondenaturing PAGE (4 °C) or treated with SDS–PAGE sample buffer and then applied to SDS–PAGE. During the course of proteolysis, aliquots (2.0 μL) were taken into buffer A (45 μL, 4 °C) containing trypsin inhibitor (5 molar ratio over trypsin) to stop the proteolysis. From this mixture, 20-μL aliquots were taken for each kinase assay.

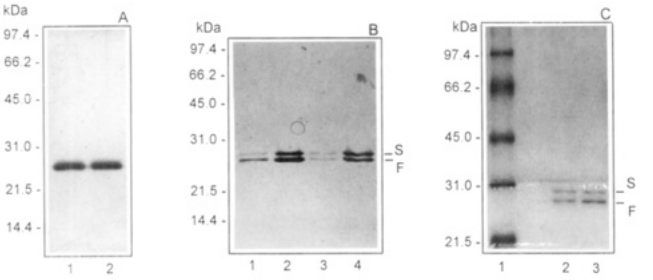

FIGURE 1: SDS–PAGE of purified kinase complex I [dCyd kinase/dAdo kinase(I)] and kinase complex II [dGuo kinase/dAdo kinase-(II)]. (A) Laemmli buffer system: lane 1, 1 μg of purified kinase complex I; lane 2, 1 μg of purified kinase complex II. (B) MZE 3328.IV buffer system (12% acrylamide gel): lane 1, 0.2 μg of purified kinase complex I; lane 2, 1 μg of purified kinase complex I; lane 3, 0.2 μg of purified kinase complex II; lane 4, 1 μg of purified kinase complex II. (C) MZE 3328.IV buffer system (12.0/13.2% acrylamide step-gradient gel): lane 1, marker proteins; lane 2, 1 μg of purified kinase complex I; lane 3, 1 μg of purified kinase complex II. F and S designate the fast- and slow-moving components, respectively.

RESULTS

Separation of Each of Two Kinase Complexes into Two Components on SDS–PAGE at pH 6.6. It was shown in our previous work that each of the affinity-purified preparations of kinase complex I (Ikeda et al., 1988) and kinase complex II (Ikeda et al., unpublished data) gave a single stained band on SDS–PAGE, using the Laemmli system (pH 8.8 gel buffer) (Laemmli, 1970), attesting to the homogeneity of each preparation. The electrophoretic mobilities of the subunits from both kinase complexes appear to be identical in this system, at 27 200 daltons (Figure 1A). Yet, when these same preparations were subjected to SDS–PAGE at a lower pH in the discontinuous MZE 3328.IV buffer system (Moos et al., 1988), two separate and distinct bands were observed in each case, as shown in Figure 1B. A step-gradient (12.0/13.2% acrylamide) long gel in the same buffer system gave even greater separation of the two components (Figure 1C). Upon comparison with protein standards, a fast-moving protein band (F-component) from both kinase complex I and kinase complex II gave an apparent mass of 27 200 daltons, which is consistent with the single band obtained with the Laemmli system, while a slower-moving band (S-component) from each enzyme complex gave an abnormally high apparent mass of 29 000 daltons. The MZE 3328.IV buffer system has been recommended by Moos et al. (1988) for SDS–PAGE in order to obtain significantly higher initial yields upon N-terminal amino acid sequencing of proteins transferred from the gel onto the PVDF membrane. The lower pH of the system (pH 6.6 gel buffer) seems to prevent N-terminal modification of the protein by reactive species remaining in the gel after polymerization. In this experiment, however, it seems likely that the heter-

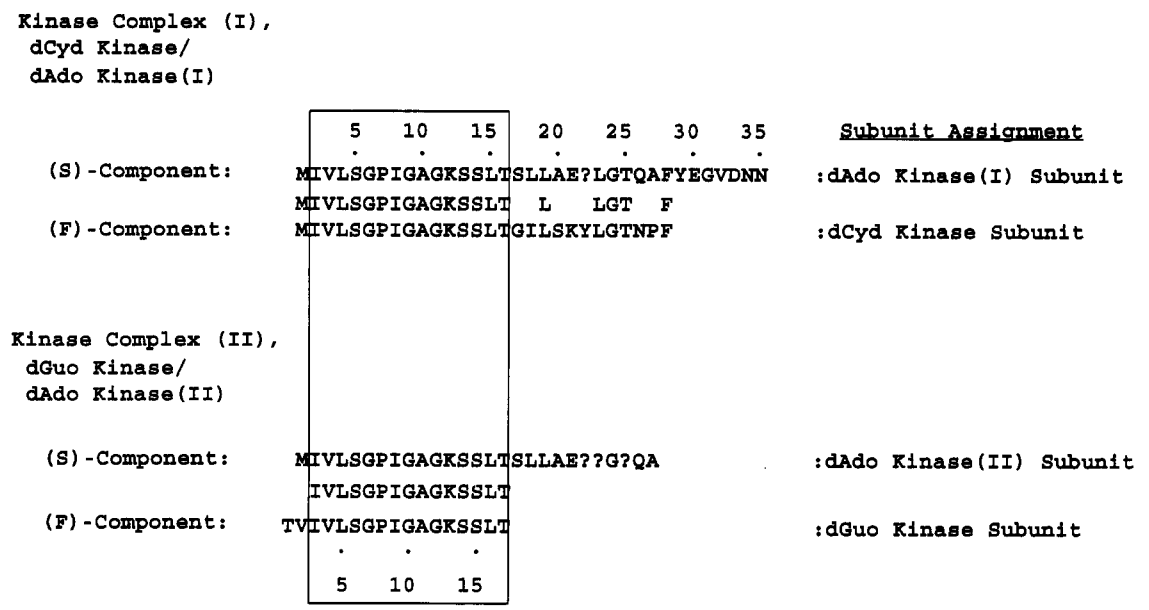

FIGURE 2: Comparison of N-terminal amino acid sequences of the subunits of kinase complex I [dCyd kinase/dAdo kinase(I)], and II [dGuo kinase/dAdo kinase(II)] from *L. acidophilus* R-26, and subunit assignment. "?" in a sequence represents a residue position where it was not possible to identify any amino acid with certainty. Matching amino acids are shown on the lines between subunit sequences.

ologous components of each enzyme pair are resolved due to variations in their binding of SDS at this lower pH.

Upon densitometric quantitation of each protein band stained with Coomassie Blue R-250, the S-component from kinase complex I was found to be about 30–80% of the F-component, depending on the enzyme preparations and/or the amount of enzyme applied to SDS–PAGE. The S-component of kinase complex II also showed 30–100% intensity compared to the F-component. Coomassie Blue is predominantly apolar, and its binding to proteins is supposed to be due to hydrophobic forces. So, under limiting conditions, different proteins (subunits) may bind different amounts of Coomassie Blue depending on their hydrophobicity or related physicochemical properties.

N-Terminal Amino Acid Sequencing of Each Component from the Two Kinase Complexes. In previous work (Ikeda et al., 1988), N-terminal sequence analysis of the intact kinase complex I protein revealed but a single amino acid species per cycle up to the 17th residue, a sequence which included the highly conserved Gly-X-X-Gly-X-Gly-Lys-Ser motif associated with ATP binding sites. At the 18th, 21st, 26th, and 27th residue positions of the sequence, however, there appeared to be 2 different amino acids in almost equal amounts. This suggested that the enzyme is composed of two nonidentical subunit polypeptides having the same amino acid sequence near the N-terminus. This has now been confirmed when the two components of kinase complex I, separated by SDS–PAGE (pH 6.6), were subjected to sequence analysis after being blotted onto the PVDF membrane (Figure 2). Quantitatively, 17.5 and 55.4 pmol of Met was detected in the first cycle for F- and S-components generated from kinase complex I (153 pmol applied to SDS–PAGE), respectively. These apparently low and scattered recoveries of N-terminal amino acids seem to be due to the combined effects of the chemical blocking of the N-terminal amino group during electrophoresis and the transfer, retention, and sequencing steps with varying efficiencies. Considering these potential variations in the recovery and analysis of the two subunits, the amount of Met detected for the first amino acid of two subunits should not be regarded as very significant in terms of subunit stoichiometry. In the later cycles of the amino acid sequence, the same pairs of amino acids previously observed at the 18th, 21st, 26th, and 27th positions are now assorted into the 2 subunits. In addition to these 4 positions, the 17th and 20th positions are also split into 2 amino acids, whereas only 1 amino acid was detected previously. It is likely that serines were overlooked at both the 17th and 20th positions in our previous work, due to technical problems in detecting small amounts of serine.

Each of two components from kinase complex II (120 pmol) has been analyzed for N-terminal sequences (Figure 2). Greater difficulty was encountered in obtaining sufficient material for sequencing, but it is shown that the N-terminus of the F-component is Thr (9.6 pmol) at the first residue and Val (10.0 pmol) at the second residue, which is quite different from the N-terminus of the S-component, Met (17.6 pmol), at the first position. This result indicates that the two bands are likely to be two distinct subunit polypeptides. However, except for this difference at the very N-terminus, the sequence from the 3rd (Ile, 11.7 pmol) to the 14th residue of the F-component exactly matches the sequence from the 2nd (Ile, 16.5 pmol) to the 13th residue of the S-component, as well as the sequences from the 2nd to the 13th residue of the 2 components of kinase complex I. Apparently, the amino acid sequence around the glycine-rich ATP-binding region is well conserved for all of these kinase subunits. By comparing the sequences of the 2 sets of subunits, it now turns out that the sequences of the slow-moving subunits from both kinase complexes are exactly the same at least to the 21st residue, i.e., to the extent they could be determined. This suggests the possibility that the S-component of each complex is the same dAdo kinase subunit associated with a different heterologous subunit, i.e., dCyd kinase or dGuo kinase.

All together, the N-terminal amino acid sequencing results indicate that kinase complexes I and II are both composed of two different subunit polypeptide chains; i.e., they are both heterodimers in quaternary structure. Therefore, it seems very likely that each subunit of the two kinase complexes would carry a single phosphorylating function for a specific deoxynucleoside substrate.

Differential Proteolysis of Two Kinase Complexes with Trypsin Controlled by the End-Product Inhibitor dNTP.

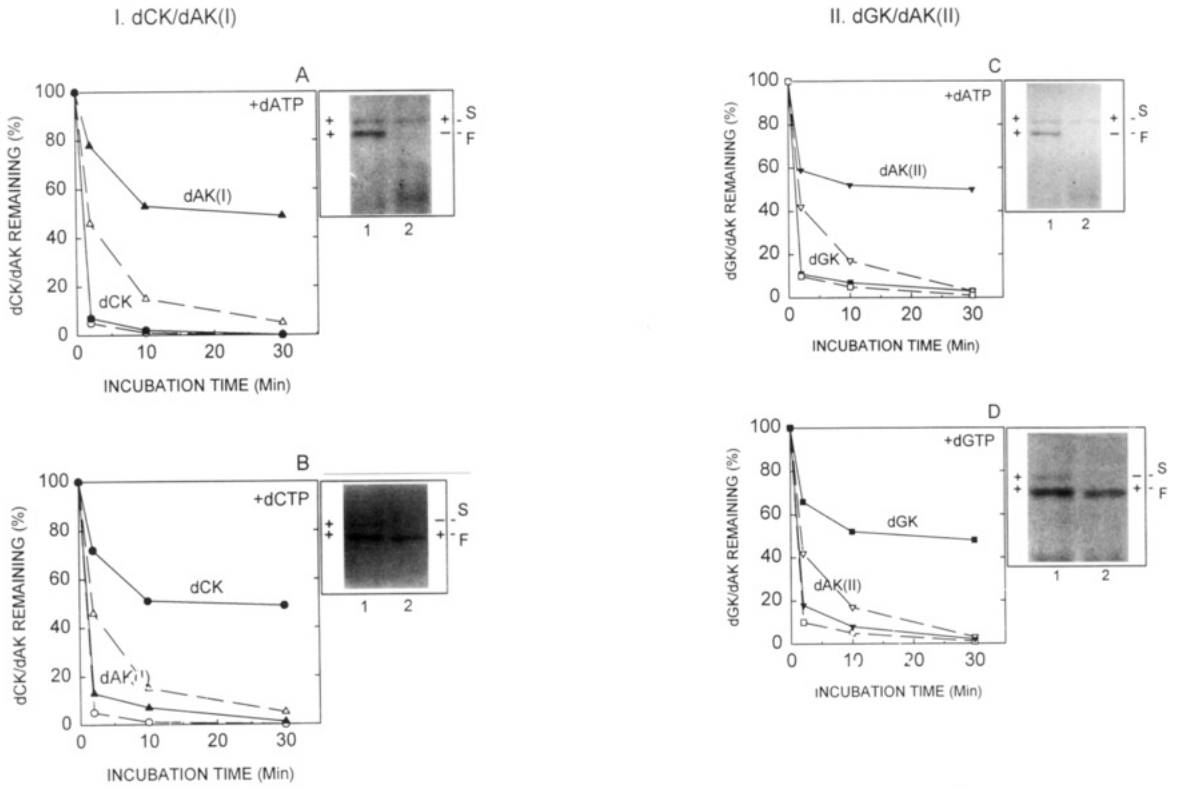

FIGURE 3: Differential limited proteolysis of kinase complex I and kinase complex II controlled by the specific end-product inhibitor dNTP. The kinase activities remaining after proteolysis with 2% (w/w) trypsin (relative to the activity at zero time) are shown: (●) dCK; (▲) dAK(I); (■) dGK; (▼) dAK(II). Kinase complex I [dCyd kinase/dAdo kinase(I)] digested in the presence of (A) 0.1 mM dATP or (B) 0.1 mM dCTP, and kinase complex II [dGuo kinase/dAdo kinase(II)] digested in the presence of (C) 0.1 mM dATP or (D) 0.1 mM dGTP. In each case, the results of a control experiment (no dNTP added) are shown by the dashed lines connecting the corresponding open symbols. Proteolysis was carried out under the conditions described under Experimental Procedures. Attached to each plot is the corresponding SDS–PAGE pattern of kinase complex I or II comparing the control and the protein differentially proteolyzed with trypsin in the presence of dNTP. Protein bands separated at pH 6.6 were stained with Coomassie Blue R-250. Lane 1, kinase complex I or II (0.5 μg, no trypsin added); lane 2, kinase complex I or II (0.5 μg) digested for 30 min in the presence of 0.1 mM dNTP. F and S designate the fast- and slow-moving subunits, respectively. In each case, the focus was put on the persistence/disappearance of the original paired subunit bands rather than on the appearance of low molecular weight proteolyzed fragments. The (+) and (–) symbols provide an interpretation of original gel patterns.

When kinase complex I or II was incubated with 2% (w/w) trypsin at 20 °C in the absence of any substrate or inhibitor, 80–95% of each kinase activity was lost within 10 min (Figure 3, dashed lines connecting open symbols in panels A and B for kinase complex I, and in panels C and D for kinase complex II), although the rate of inactivation of both dAdo kinases appears to be significantly lower than that of either counterpart kinase. Apparently, any of the paired kinase subunits in the native state is subject to proteolysis by trypsin when its active site is not occupied by any ligand. On the other hand, when 10 mM ATP was added to the digestion mixture, all kinase activities appeared to be nonspecifically protected from proteolysis (at most 10% inactivation by 10-min incubation in each case, not shown), reflecting the simultaneous binding of saturating ATP at the two phosphorylation sites of each kinase complex.

Next, in order to see if the specific end-product inhibitor, dNTP, can preferentially protect the corresponding kinase subunit against proteolysis, each dNTP was added to a digestion mixture. The concentration of dNTP (0.1 mM) was chosen to saturate the binding site for each dNTP [the K_i for each dNTP is around 1 μM or less (Ikeda et al., 1986)]. As shown in four separate sections of Figure 3, one member of the paired kinases—the one not protected by its corresponding dNTP—remains susceptible to trypsin and loses 80–90% of its activity within 2 min. In other words, a dNTP contributes almost no protective effect on its counterpart kinase activity; rather, dCTP or dGTP seems even to stimulate the rate of inactivation of the associated dAdo kinase. On the other hand, the kinase component protected by its corresponding dNTP retains about half of its original activity, even after 30 min of incubation. About the same level of activity remains after 30 min even when the amount of trypsin was increased to 10% of the amount of kinase (results not shown). Concurrent with the almost total loss of one of the paired kinase activities, one of the original paired protein bands revealed on SDS–PAGE at pH 6.6 completely disappears by 30 min of digestion (Figure 3). The other band appears to be unchanged, although exact quantitation has not been done. Further analysis on SDS–PAGE (15% acrylamide, pH 8.8) (Figure 4) revealed only two clear fragments (15.8 and 11.0 kDa) following proteolysis of kinase complex I with 2% trypsin for 3 min in the presence of dATP (corresponding to Figure 3A). The ratio of 15.8-kDa and 11.0-kDa fragments stayed almost the same with the digestion time extended to 10 min. By comparing the results obtained with SDS–PAGE at pH 8.8 (Figure 4) and pH 6.6 (Figure 3A, lane 2), it appears likely that two fragments on Figure 4 are derived from the original fast-moving subunit and the protein band remaining at 27.2 kDa is the original slow-moving subunit. This result may indicate that the kinase polypeptide chain not protected by dNTP is cut by trypsin at a single specific site, with concomitant loss of activity. On the other hand, judging from the persistence of the remaining activity (around 50%) and protein band density after prolonged incubation, any one of subunits appears to become fully resistant to trypsin when its active site is occupied by its homologous dNTP. The initial rapid drop in the activity of the protected subunit may be due

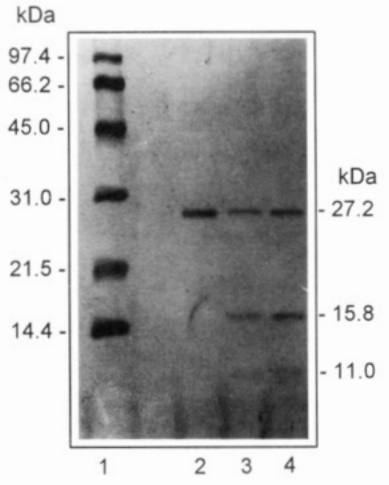

FIGURE 4: Proteolytic fragments separated on SDS–PAGE (15% acrylamide gel, pH 8.8). Kinase complex I [dCyd kinase/dAdo kinase(I)] was incubated with 2% trypsin in the presence of 0.1 mM dATP. The condition for proteolysis corresponds to those for Figure 3A. The digestion was stopped by adding PMSF. Lane 1, marker proteins (1 μg each); lane 2, kinase complex I (1 μg, no trypsin added); lane 3, kinase complex I digested for 3 min; lane 4, kinase complex I digested for 10 min.

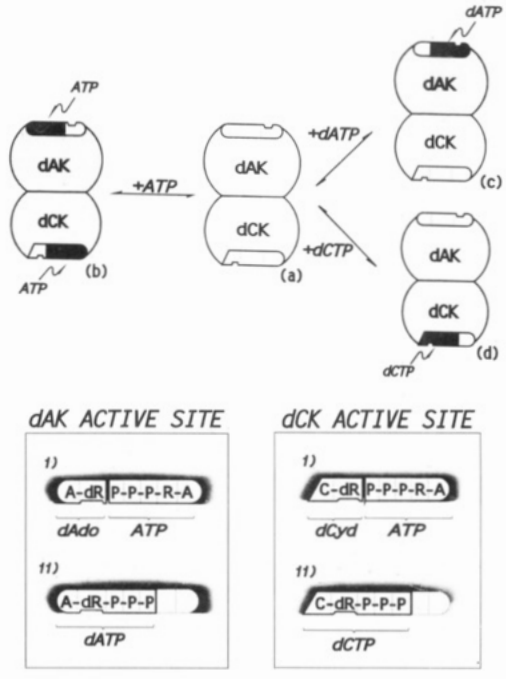

FIGURE 5: (Top) Schematic representation of heterodimers of kinase complex I [dCyd kinase/dAdo kinase(I), dCK/dAK] and kinase complex II [dGuo kinase/dAdo kinase(II), dGK/dAK] as implicated from differential limited proteolysis controlled by the end-product inhibitor dNTP. The heterodimer model in this figure represents kinase complex I, but it could also represent kinase complex II because of its nearly identical characteristics. The shape and size of each subunit and its active site carry no real dimensions, but the diagram is an attempt to incorporate into a visual model the previous kinetic results on the binding site for dNTP in relation to the binding site for substrates and the present results on the differential proteolysis of each kinase subunit. The shaded subunits are protected, while the white subunits are susceptible to limited proteolysis. The bottom panel represents a more detailed model of the active site of each deoxynucleoside kinase and putative modes of binding of substrates and dNTP, as described in our previous work (Ikeda et al., 1986). Symbols: A, adenine; C, cytosine; R, ribose; dR, deoxyribose; P, phosphate group. (i) Substrates (deoxynucleoside and MgATP); (ii) dNTP.

to a conformational effect resulting from proteolysis of the counterpart subunit, or, perhaps, to the dissociation of fragments derived from the proteolyzed subunit. In a preliminary experiment using nondenaturing PAGE (12% acrylamide), a kinase complex I preparation differentially proteolyzed in the presence of dATP showed a slightly greater mobility compared with the untreated enzyme (not shown). Further investigation is necessary to determine whether the fragments from the proteolyzed subunit stay associated with the counterpart subunit or if they dissociate. Overall, the dNTPs seem to be effective ligands with which to protect specific kinase subunits from tryptic proteolysis, while leaving the heterologous kinase subunit in each case susceptible to it. When an even higher concentration (0.2 mM) of dNTP was added to the digestion mixture (not shown), its protective effect on the corresponding kinase remained unchanged, whereas the inactivation of the counterpart kinase was slowed down significantly. As the dNTP concentration approaches the range of dissociation constants for phosphate donors like ATP, the dNTP may bind nonspecifically to the phosphate donor sites in the same orientation as ATP. This is in contrast to its specific and much tighter binding as an inhibitor at the active site of its corresponding kinase, where it is presumably positioned in a reversed orientation relative to ATP (Deibel et al., 1977; Ikeda et al., 1988). Therefore, 0.1 mM dNTP seems to be the most suitable concentration for the differential proteolysis of each kinase.

In any event, for the purpose of this paper, these results enable us to clearly assign the phosphorylation function for a particular deoxynucleoside substrate to each subunit of the two kinase complexes. Thus, we relate the F- and S-components of kinase complex I to the dCyd kinase subunit and the dAdo kinase(I) subunit, respectively (Figure 2). Similarly, the F- and S- components of kinase complex II are now assigned to the dGuo kinase subunit and the dAdo kinase-(II) subunit, respectively.

DISCUSSION

It is now evident that both kinase complex I [dCyd kinase/dAdo kinase(I)] and kinase complex II [dGuo kinase/dAdo kinase(II)] from *Lactobacillus acidophilus* R-26 are heterodimers carrying distinct phosphorylation sites for two deoxynucleoside substrates on separate subunits, as depicted in Figure 5. This model is intended to reflect the following characteristics of the kinase complexes: (1) Each of the paired subunits has a similar molecular size (~27 200 daltons) (Ikeda et al., 1988, unpublished data) with a certain symmetry between two subunits, such as the presence of the consensus sequence for an ATP binding site near the N-terminus of each subunit (Figure 2). (2) At least one distinct active site for a specific deoxynucleoside substrate is located on each subunit. Such specificity is represented by different shapes for the dCyd and dAdo binding sites. On the other hand, the ATP binding site of each subunit is drawn in the same shape in spite of a difference in their K_ms. (3) The binding sites for deoxynucleoside and ATP must be situated next to each other at each active site, according to its sequential kinetics (Deibel et al., 1977; Chakravarty et al., 1984). (4) The end-product inhibitor, dNTP, specifically and strongly inhibits the corresponding kinase activity by binding to each catalytic site (Deibel & Ives, 1977a; Ikeda et al., 1986). Presumably, the deoxynucleoside moiety of dNTP fits into the deoxynucleoside binding site, while the triphosphate portion of dNTP interacts with the triphosphate binding site for ATP, but in a reversed orientation relative to that of ATP (see the bottom panel of Figure 5) (Ikeda et al., 1986). (6) Each subunit becomes resistant to trypsin upon the binding of dNTP or ATP to its

active site, as demonstrated in this work. This change in proteolytic susceptibility is reflected by shading on the respective subunit.

The existence of such an unusual organization of the deoxynucleoside kinase activities in the quaternary structures of the two kinase complexes was strongly implicated by the rather surprising resolution of each kinase complex into two components by SDS–PAGE at pH 6.6 (Figure 1) and by the distinct N-terminal amino acid sequence subsequently found for each component (Figure 2). Isoelectric focusing in the presence of urea also gave paired major bands for each of the two kinase complexes (results not shown). However, several minor additional bands were observed probably due to deamidation or the formation of protein–Ampholite artifacts. Since the separation of subunits on SDS–PAGE at pH 6.6 has turned out to be so effective, as well as technically very simple, no other method for resolving the two subunits has been sought. The direct demonstration of a different phosphorylation site on each distinct subunit, i.e., the assignment of a specific kinase function to each subunit separated on SDS–PAGE, has been achieved by means of the remarkable specificity of the differential limited proteolysis of each subunit controlled by the respective end-product inhibitor, dNTP (Figure 3). The possibility of homodimers, in which each subunit carries phosphorylating functions for both dCyd and dAdo (or dGuo and dAdo) on a single polypeptide chain, has clearly been ruled out.

Upon addition of the corresponding dNTP, each kinase subunit appears to become differentially stable to trypsin (Figure 3). Since it has been inferred from kinetics that dNTP binds to the active site of the corresponding kinase (Ikeda et al., 1986), it is reasonable to assume that the protection of each kinase from proteolysis is the result of the specific dNTP bound to the active site (Figure 5c,d). This differential protection rendered by dNTP contrasts markedly with the nondifferential protection shown by ATP (Figure 5b), the effect of either nucleotide reflecting its individual binding specificity. Since a similar degree of protection was observed with saturing concentrations of dNTP and ATP, it is attractive to assume that a susceptible portion of the polypeptide is present within the triphosphate binding site and is protected either by dNTP or by ATP and that, in the absence of any ligand, proteolytic cleavage at this essential site renders the kinase subunit inactive. This phenomenon of protection of an enzyme from proteolysis upon the binding of substrate or inhibitor has been observed in many instances (Mihalyi, 1972), and in some cases, the change in conformation accompanying ligand binding can be quite drastic (Trayser & Colowick, 1961). However, strictly speaking, it has yet to be determined whether the kinase subunit is protected from proteolysis simply by the physical or steric interference of the bound dNTP, or if the susceptible portion of the polypeptide is remote from the site of dNTP binding, but becomes unavailable to trypsin due to the resulting conformational change.

In addition to its protective effect on the corresponding kinase subunit, dCTP or dGTP appears to stimulate the proteolysis of the associated dAdo kinase (Figure 3B,D). It has been shown in our previous work that dCTP (or dGTP) stimulates the dAdo kinase activity in kinase complex I or II 3–5-fold, when assayed with a subsaturating concentration of ATP (Deibel et al., 1977; Chakravarty et al., 1984). These two phenomena, the activation of dAdo kinase activity and the stimulation of proteolysis of dAdo kinase subunit, appear to be coupled to the conformational change induced by the binding of dCTP (or dGTP) to the dCyd kinase (or dGuo kinase) subunit. This subject will be discussed in detail somewhere else in terms of subunit–subunit interaction. In any event, compared with the rather common phenomenon of protection of an enzyme from proteolysis by ligands, the stimulation of proteolysis upon the binding of some ligand to an enzyme is unusual, but it is a clear-cut observation which directly shows a conformational change induced by ligand binding. Such increased proteolytic susceptibility has also been found in the case of aspartate transcarbamylase upon the binding of its substrate, aspartate (McClintock & Markus, 1968). In contrast to the stimulatory effect of dCTP (or dGTP) on dAdo kinase, no such effect with dATP was observed on the proteolysis of dCyd kinase (or dGuo kinase) (Figure 3A,C), consistent with the kinetic observation that the addition of dATP produces no remarkable effect on the activity of the associated dCyd kinase (or dGuo kinase) (Deibel et al., 1977; Chakravarty et al., 1984).

Each of the N-terminal amino acid sequences obtained for the two subunits separated on SDS–PAGE (pH 6.6) is now assigned to a particular kinase subunit, as shown in Figure 2. Although the sequence data are limited, especially in the case of the dGuo kinase subunit, as far as it can be determined the N-terminal amino acid sequences of all of the subunits look very similar to one another. All 4 subunits carry a common 15-residue sequence [2nd–16th amino acid for the dCyd kinase subunit, the dAdo kinase(I) subunit, and the dAdo kinase(II) subunit; 3rd–17th amino acid for the dGuo kinase subunit], which includes the consensus GXXGXGKS sequence for an ATP binding site, at the very N-terminus. The dGuo kinase subunit is found to possess a unique N-terminus (Thr-Val) just preceding this common sequence portion, compared with a methionine initiating the N-termini of the other three kinase subunits. It is interesting that previous kinetic experiments have revealed that dGuo kinase exhibits an unusually high K_s for ATP (1700 μM) (Deibel et al., 1977; Chakravarty et al., 1984), compared with the other three kinases ($\sim$100 μM) (Deibel et al., 1977; Chakravarty et al., 1986). Further investigation is necessary to see if these two findings are related. In contrast to broad specificity for the phosphate donor, each kinase shows almost absolute deoxynucleoside substrate specificity (Deibel & Ives, 1977a). The structural basis for this specificity must be found in the internal amino acid sequence of each kinase polypeptide. As far as the sequences can be read, the dAdo kinase(I) and dAdo kinase(II) subunits share a common amino acid sequence at the 1st–21st, 25th, 27th, and 28th residues, without exception. These two dAdo kinase subunits have identical electrophoretic mobilities on SDS–PAGE at both pH 8.8 and pH 6.6 (Figure 1). Previous work has also shown that these two dAdo kinases exhibit very similar kinetic properties, including an ordered kinetic mechanism, nearly identical specific activities, and K_m values for dAdo and ATP, and that both show specific inhibition by dATP and activation by substrates of the opposite subunit (Deibel et al., 1977; Chakravarty et al., 1984). Further study is necessary to establish whether or not these two dAdo kinases are the products of one gene and are associated randomly with the dCyd kinase or dGuo kinase subunit, or if two paired kinases are produced in tandem from totally separate genetic sequences. Cloning and sequencing of the genes responsible for these two kinase complexes are now in progress in our laboratory.

In our previous work, the specific activity of each kinase subunit from kinase complex I and II purified to homogeneity has been found to be quite consistent under standard assay conditions for a number of enzyme preparations: dCK, 2540

units/mg; dAK(I), 360 units/mg (Ikeda et al., 1988); dGK, 2150 units/mg; dAK(II), 280 units/mg (Ikeda et al., unpublished data). Apparently, dAdo kinase is much less active than the respective counter-kinase. However, both dAdo kinase(I) and dAdo kinase(II) are activated 5–7 times by the presence of the deoxynucleoside substrate for the counter-subunit (dCyd and dGuo, respectively) (Deibel et al., 1977; Chakravarty et al., 1984). In the opposite direction, stimulation of dCyd kinase and dGuo kinase is much less significant with, at most, a 20% increase produced by the presence of dAdo. Thus, in the fully activated state, all four kinase subunits combined express very similar specific activities. The 1:1 stoichiometry between the two subunits in each kinase complex was suggested by the almost equal amounts of two distinct amino acids detected at certain cycles of sequencing when the intact kinase complex I was analyzed (Ikeda et al., 1988). However, it has not been clearly demonstrated either by quantitation of stained bands on SDS–PAGE or by amino acid sequencing of each subunit, probably because of physicochemical differences between the two subunits, as discussed briefly under Results. The abnormally slow mobility on SDS–PAGE at pH 6.6 and the relatively weak intensity of the stained band (Figure 1B) may be related to the same structural features of the slow-moving dAdo kinase subunit of each complex. The possibility of the presence of, and the contamination by, the homodimer in which two dCyd kinase (or dGuo kinase) subunits are associated is not totally excluded, but this appears highly unlikely in light of previous experiments in which we used a series of affinity columns specific for one of the kinases. For example, all the dCyd kinase activity present in the crude extract of *Lactobacillus* cells was bound to a dAp_4A–Sepharose column and eluted together with dAdo kinase by addition of dATP (Ikeda & Ives, 1985), proving that there is no dCyd kinase, either as monomer, as homodimer, or as heterodimer, which is not associated with dAdo kinase. Similarly, almost all dGuo kinase activity in the crude extract was bound to dATP–Sepharose and eluted together with dAdo kinase by addition of dATP (Ikeda et al., unpublished data). It seems that only asymmetric association into the existing dimeric combinations is allowed and that the known heterodimeric structures, once formed, seem to be quite stable. Heterodimeric enzymes containing subunits catalyzing the first committed step of parallel metabolic pathways appear to be very rare, and the heterodimeric structures of the two deoxynucleoside kinase complexes of *Lactobacillus acidophilus* R-26 may even be unique. There may be some reason in terms of stability for heterodimer formation. However, it is not clear why the regulation of dAdo kinase activity by the substrate or inhibitor of the countersubunit is required, nor why dAdo kinase is associated with both dCyd and dGuo kinases, while thymidine kinase is not (Durham & Ives, 1971). It would be interesting to know whether a dissociated monomer is active or not. While it is difficult to dissociate subunits without the use of denaturing reagents, perhaps the extensive proteolysis of one subunit may yield the other subunit as a monomer retaining some activity. It is not yet clear whether associated proteolyzed fragments of one subunit are essential for the activity of the other subunit.

ACKNOWLEDGMENT

We are grateful to Dr. Richard Swenson for critical reading of the manuscript.

REFERENCES

Bradford, M. (1978) *Anal. Biochem. 72*, 248–254.
Chakravarty, R., Ikeda, S., & Ives, D. H. (1984) *Biochemistry 23*, 6235–6240.
Deibel, M. R., & Ives, H. D. (1977a) *J. Biol. Chem. 252*, 8235–8239.
Deibel, M. R., & Ives, H. D. (1977b) *Methods Enzymol. 51*, 346–354.
Deibel, M. R., Jr., Reznik, R. B., & Ives, H. D. (1977) *J. Biol. Chem. 252*, 8240–8244.
Durham, J. P., & Ives, D. H. (1971) *Biochim. Biophys. Acta 228*, 9–25.
Ikeda, S., & Ives, D. H. (1985) *J. Biol. Chem. 260*, 12659–12664.
Ikeda, S., Chakravarty, R., & Ives, D. H. (1986) *J. Biol. Chem. 261*, 15836–15843.
Ikeda, S., Swenson, R. P., & Ives, D. H. (1988) *Biochemistry 27*, 8648–8652.
Ives, D. H. (1984) *Anal. Biochem. 136*, 416–420.
Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods 10*, 203–209.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
McClintock, D. K., & Markus, G. (1968) *J. Biol. Chem. 243*, 2855–2862.
Mihalyi, E. (1972) in *Application of Proteolytic Enzymes to Protein Structure Studies*, pp 151–155, CRC Press, Boca Raton, FL.
Moos, M., Jr., Nguyen, N. Y., & Liu, T.-Y. (1988) *J. Biol. Chem. 263*, 6005–6008.
Speicher, D. W. (1989) in *Techniques in Protein Chemistry* (Hugli, T. E., Ed.) pp 24–35, Academic Press, Inc., New York.
Trayser, K. A., & Colowick, S. P. (1961) *Arch. Biochem. Biophys. 94*, 169–176.