A Triglobular Model for the Polypeptide Chain of Aspartokinase I-Homoserine Dehydrogenase I of *Escherichia coli*†

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ABSTRACT: Limited proteolysis of aspartokinase I-homoserine dehydrogenase I from *Escherichia coli* by type VI protease from *Streptomyces griseus* yields five proteolytic fragments, three of which are dimeric, the other two being monomeric. One of the monomeric fragments (27 kilodaltons) exhibits residual aspartokinase activity, while the second one (33 kilodaltons) possesses residual homoserine dehydrogenase activity. The smallest of the dimeric species (2 × 25 kilodaltons) is inactive; the two other dimers exhibit either only homoserine dehydrogenase activity (2 × 59 kilodaltons) or both activities (hybrid fragment, 89 + 59 kilodaltons). This characterization of the proteolytic species in terms of molecular weight, subunit structure, and activity leads to the proposal of a triglobular model for the native enzyme. In addition, the time course of the formation of the various fragments was followed by measuring enzymatic activity and performing gel electrophoretic analysis of the protein mixture at defined time intervals during proteolysis. On the basis of the results of these studies, a reaction scheme describing the succession of events during proteolysis is given.

Aspartokinase I-homoserine dehydrogenase I (AK I-HDH I)[1] from *Escherichia coli* K12 is a bifunctional protein which catalyzes two nonconsecutive reactions in the biosynthetic pathway leading from aspartate to threonine (Patte et al., 1966; Truffa-Bachi et al., 1968; Cohen & Dautry-Varsat, 1980). Its synthesis and activities are regulated by L-threonine (Stadtman et al., 1961; Patte et al., 1963). It is made up of four identical polypeptide chains of molecular weight 89 000, the amino acid sequence of which has been recently determined (Falcoz-Kelly et al., 1972; Katinka et al., 1980). The sites involved in both the kinase and dehydrogenase activities are present in the same chain, albeit in different regions: the kinase activity is carried by the N-terminal moiety and the dehydrogenase activity by the C-terminal moiety (Véron et al., 1972). This has been established by obtaining two smaller fragments endowed with only one of the two activities: limited proteolysis by a variety of proteases (Véron et al., 1972; Sibilli et al., 1981) yields a homodimeric fragment (2 × 56–57 kilodaltons) which has only the dehydrogenase activity, the fragment produced by subtilisin having the same C-terminal end as AK I-HDH I (Véron et al., 1972); on the other hand, another fragment (4 × 53 kilodaltons) (Janin et al., 1967; Sibilli et al., 1982) with the same N-terminal end as AK I-HDH I can be extracted from an *ochre* mutant (Thèze & Saint-Girons, 1974) and possesses only the kinase activity (Véron et al., 1972). These two fragments correspond to partially overlapping segments of the original polypeptide chain, and it has not been possible yet to derive a set of nonoverlapping fragments from AK I-HDH I.

Limited proteolysis of large proteins has often provided useful information about their function and spatial structure. The isolation and extensive characterization of the fragments produced by limited proteolysis may indeed reveal the existence of several compact regions, the role of each of these regions in the biological activity of the intact protein, the importance of the mutual interactions between these regions inside the whole assembly, etc. We have reinvestigated in more detail the limited proteolysis of AK I-HDH I, using Pronase, a nonspecific protease from *Streptomyces griseus*, to cleave the polypeptide chain. The present report describes the isolation of the various fragments and their characterization in terms of enzymatic activity, size of the polypeptide chains, and subunit structure. The spectrum of fragments and their properties which is consistent with our previous knowledge of AK I-HDH I, can be used to refine our model of the organization of the native enzyme (Véron et al., 1973).

Materials and Methods

Buffers, Chemicals, Enzymes, and Activity Measurements. Buffer A contains 20 mM potassium phosphate, pH 7.2, 0.15 M KCl, 0.2 mM magnesium titriplex, 2 mM L-threonine, and 1 mM dithiothreitol. Buffer P is buffer A without KCl and threonine.

The chemicals used were all of analytical grade and were purchased from Sigma Chemical Co. or Merck Darmstadt. Pyruvate kinase, lactate dehydrogenase, and protease type VI from *S. griseus* were from Sigma (Sigma has discontinued production of type VI, but a comparable substitute may be type XIV). Acrylamide was obtained from Serva. Low and high molecular weight markers for the calibration of columns and gels were obtained from Pharmacia Fine Chemicals.

AK I-HDH I from *E. coli* K12, strain Tir8, was purified to homogeneity as previously described (Janin & Cohen, 1969). The dehydrogenase and kinase activities were measured as already published (Truffa-Bachi & Cohen, 1970). AK I-HDH I was stored in buffer A at room temperature as an ammonium sulfate precipitate (50% saturation).

Proteolysis and Purification of Proteolytic Fragments. The precipitate was centrifuged at 20000*g* for 30 min. The pellet was resuspended in buffer P and dialyzed overnight against the same buffer. Proteolysis was carried out in buffer P at 27 °C; the concentration of the protease ranged from 0.5 to 1.0% w/w. Unless otherwise stated, the concentration of AK I-HDH I was 0.5 mg/mL. The progress of proteolysis was monitored by measuring the decrease in enzymatic activities and the desensitization of HDH activity to threonine. The reaction was terminated by addition of 2 mM phenyl-

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; magnesium titriplex, K_2Mg salt of ethylenediaminetetraacetic acid; AK, aspartokinase; HDH, homoserine dehydrogenase; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase.

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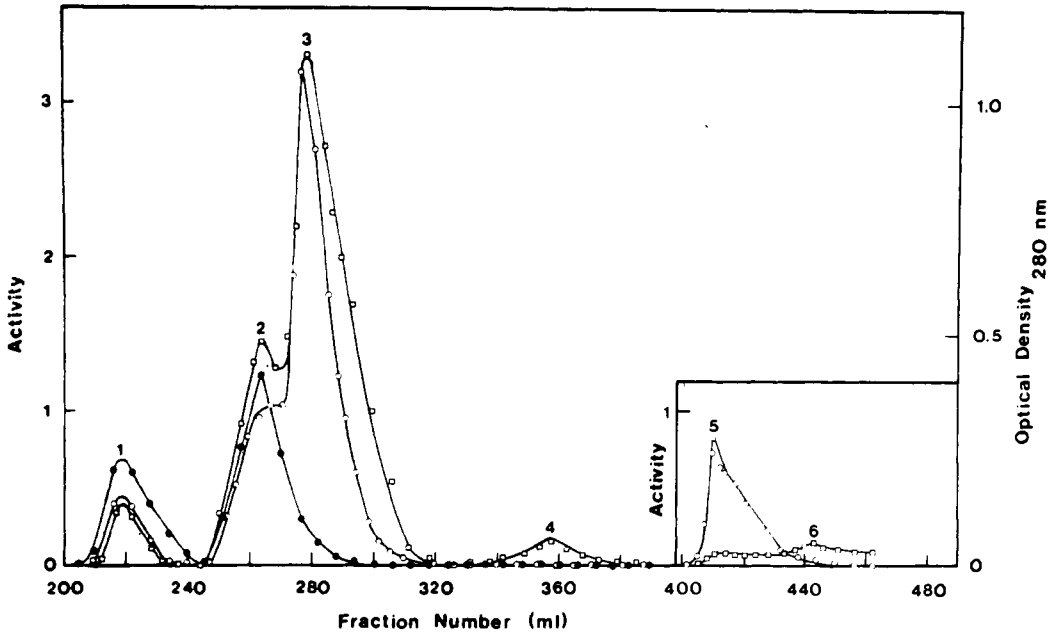

FIGURE 1: Separation of the proteolytic fragments. The proteolysis of 100 mg of AK I–HDH I (protease:AK I–HDH I ratio 0.5% w/w) was terminated when 21% AK activity and 65% HDH activity had remained, corresponding to 105-min incubation at 27 °C. The protein mixture was concentrated, resuspended in 5.8 mL of buffer A, and applied onto an Ultrogel ACA 34 column (2.5 cm × 90 cm). Fractions were assayed for AK (●) and HDH (○) activities. One unit of HDH activity corresponds to 10 μmol of NADPH min^{-1} mL^{-1}, while 1 unit of AK activity is expressed as 1 μmol of NADH min^{-1} mL^{-1}. The absorbance of the proteins (□) was measured at 280 nm. The inset represents the activity in peak 5, which is expressed as 1 μmol of NADPH min^{-1} mL^{-1}.

methanesulfonyl fluoride, which inhibits serine proteases irreversibly (Gold & Fahrney, 1964). At the termination of proteolysis, 2 mM L-threonine and 150 mM KCl were also added.

After preparative proteolysis, the resulting protein mixtures (250–300 mL) were precipitated by 50% ammonium sulfate; after centrifugation, at 20000*g*, the pellet was resuspended in 7–10 mL of buffer A. The protein concentrate was applied onto an Ultrogel ACA 34 column (2.5 cm × 90 cm) equilibrated with buffer A. The proteins were eluted from the column with buffer A at a flow rate of 5 mL/h. The purified protein species were pooled and stored as ammonium sulfate precipitates (50%). The apparent molecular weights of the eluted species were estimated by their elution volume after calibration of the column with the following protein standards: catalase 232000, lactate dehydrogenase 144000, alcohol dehydrogenase 70000, and chymotrypsinogen A 25000.

Electrophoresis. Polyacrylamide gels (7.5%) were prepared according to Davis (1964). The gels and the electrophoresis buffer contained 2 mM L-threonine. Gels were run in Tris–glycine buffer pH 8.3 at a constant current of 2 mA/tube and were stained with Coomassie blue type G (0.02% in 3.5% perchloric acid) or with Coomassie blue type R (0.1% in 50% methanol and 10% acetic acid). The gels were destained either by 7.5% acetic acid or by 25% methanol–7.5% acetic acid (Wilson, 1979). HDH activity was revealed on the gels according to Truffa-Bachi et al. (1968). $NaDodSO_4$ slab gels (10%) (Laemmli, 1970) were stained with Coomassie blue type R and destained with 30% methanol containing 7% acetic acid. The subunit molecular weight of the fragments were determined by measuring their R_f and comparing them with the values for proteins of known molecular weights. The following polypeptides were used: phosphorylase *b* 94000, bovine serum albumin 67000, ovalbumin 43000, carbonic anhydrase 30000, and soybean trypsin inhibitor 20000.

Results

Separation of Proteolytic Fragments. Figure 1 shows the elution profile obtained upon gel filtration of the mixture of protein species produced in a typical limited proteolysis of AK I–HDH I. Six peaks can be identified and are numbered from 1 to 6 in the order of their elution. The same six peaks, but with different relative sizes, elute at the same volumes in other experiments where different conditions of proteolysis (time and/or protease concentration) are used. Thus, the species present in these six peaks define a characteristic spectrum of the fragments derived from AK I–HDH I by limited proteolysis. In all cases, the principal portion of total protein is found in the three major peaks, 1, 2, and 3, whereas the three minor peaks, 4, 5, and 6, always represent only a small part of the total protein. For this reason, some of the properties of the species present in peaks 4–6 had to be studied either after concentrating them on Amicon PM10 membranes or after using different conditions of proteolysis to increase their amount (see later).

At least one of the enzymatic activities (kinase or dehydrogenase) can be measured in five out of these six protein peaks. Both activities are present in peaks 1 and 2. Peak 3 contains mainly dehydrogenase activity and also some contaminating kinase activity as the result of the incomplete separation between peaks 2 and 3. Neither kinase nor dehydrogenase activity can be detected in peak 4. Peak 5 shows only some dehydrogenase activity and peak 6 only some kinase activity at a low but detectable level.

The number and/or homogeneity of the protein species present in each peak can be analyzed by performing electrophoresis on polyacrylamide gels under nondenaturing conditions on selected fractions of the elution profile shown in Figure 1. Peak 1 contains a single protein species (Figure 2a). Peaks 2 and 3 are not completely resolved; however, some fractions exist where each protein species is present in an homogeneous state (Figure 2b,c). Peak 4 contains two different protein species. Peaks 5 and 6 are discussed below, because their analysis involves protein concentration.

Characterization of Proteolytic Fragments. The column used in Figure 1 has been calibrated with proteins of known

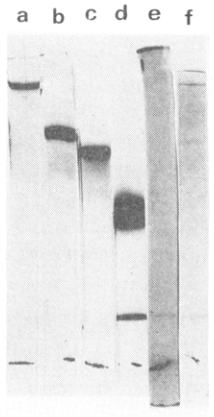

FIGURE 2: Polyacrylamide gel electrophoresis under native conditions of selected fractions of the Ultrogel ACA 34 column described in Figure 1. A 10-μL sample of fraction 216 [(a) AK I-HDH I], 255 [(b) H], and 300 [(c) HDH] and a 50-μL sample of fraction 354 [(d) I_D], 311 [(e) HDH_M], and 442 [(f) AK_M] were analyzed. The bands in lanes e and f, although weak, are perfectly visible on the actual gels.

molecular weight, providing a measurement of the molecular weight of each fragment under nondenaturing conditions. Although this determination of molecular weight from the elution volume is somehow approximate, it does not lead to any ambiguity in the conclusions relative to the monomeric or dimeric nature of the fragments obtained. The various fragments were also characterized by their enzymatic properties. Kinase and dehydrogenase activities were assayed in the standard conditions used for AK I–HDH I. Dehydrogenase activity was also monitored directly on polyacrylamide gels (see Materials and Methods). Inhibition by L-threonine of enzymatic activities was studied in the case of the homogeneous species present in the major peaks 1–3. The molecular weight(s) of the polypeptide chain(s) present in each fragment was (were) determined from the electrophoretic mobilities on polyacrylamide gels in the presence of $NaDodSO_4$. Below are given the results obtained with the six peaks already described.

(*a*) *Peak 1*: *Uncleaved Native AK I–HDH I.* Peak 1 (Figure 1) contains a single species (Figure 2a) which is identical with native AK I–HDH I by the following criteria: (i) its native molecular weight, around 360 000; (ii) the molecular weight of its chains, 90 000; (iii) the presence of the two activities and their level with respect to absorbance (Figure 1); (iv) the usual cooperative inhibition of these two activities by L-threonine.

(*b*) *Peak 2*: *A Hybrid Fragment, H, Composed of Two Major Chains of Different Sizes.* The protein present in peak 2 is homogeneous (Figure 2b) and has an apparent molecular weight around 170 000 under native conditions. Upon gel electrophoresis in the presence of $NaDodSO_4$, this protein gives two major bands (Figure 3a) corresponding to molecular weights of 89 000 and 59 000. This fragment is therefore composed of two major chains of different sizes and will be called H fragment (for hybrid) from here on. The fact that the apparent molecular weight of 170 000 is slightly higher than the sum 89 000 + 59 000 = 148 000 may indicate that fragment H has an elongated shape.

Analysis of a mixture of native AK I–HDH I and H fragment on $NaDodSO_4$ gels shows that the 89 000 chain still migrates as a single band and thus that this chain has not been appreciably cleaved upon formation of H.

The H fragment possesses both kinase and dehydrogenase activities; also, these activities are inhibited by L-threonine but not with the same features as native AK I–HDH I. The presence of 10^{-4} M L-threonine hardly affects the kinase activity of AK I–HDH I while inhibiting the kinase activity of H by 50%. In contrast, the presence of 2×10^{-3} M L-threonine inhibits the dehydrogenase activity of AK I–HDH I by 75% and that of H by only 35%.

(*c*) *Peak 3*: *A Dimeric Homoserine Dehydrogenase Fragment, HDH_D.* Peak 3 contains a single protein species (Figure 2c) with an apparent molecular weight under native conditions around 115 000. A single species, corresponding to a molecular weight of 59 000, is obtained upon electrophoresis in the presence $NaDodSO_4$. This fragment is thus a dimer, composed of two chains of equal size. It possesses a dehydrogenase activity which is not inhibited by threonine. This fragment, called HDH_D, is therefore very similar to the species obtained previously with several other proteases and which has been extensively studied in the case of subtilisin cleavage of AK I–HDH I (Vĕron et al., 1972). HDH_D is the more stable fragment observed during proteolysis and it has been repeatedly observed (Vĕron et al., 1973).

(*d*) *Peak 4*: *An Inactive Dimeric Fragment, I_D.* Peak 4 corresponds to an apparent molecular weight of around 53 000, as determined by its elution volume. On $NaDodSO_4$–polyacrylamide gels, a single protein band with a molecular weight of 25 000 is observed, showing that the species present in peak 4, called I_D fragment, has a dimeric structure composed of two chains of equal size. The fact that I_D fragment exhibits two bands on a native gel (Figure 2d) probably arises from a partial dissociation of the dimer into monomers (see below). It is devoid of either enzymatic activity.

(*e*) *Peak 5*: *A Monomeric Homoserine Dehydrogenase Fragment, HDH_M.* When gel electrophoresis is carried out under native conditions on one fraction of peak 5, a single protein band is observed (Figure 2e). This single band has dehydrogenase activity, which can be detected directly on the gel (not shown). In order to study the material present in peak 5 which is quite dilute, the active fractions were pooled and concentrated 40-fold with an Amicon PM10 membrane. Gel electrophoresis of the concentrated protein in the presence of $NaDodSO_4$ reveals three bands with molecular weights of 33 000, 43 000, and 45 000 (not shown). This shows that peak 5 is heterogeneous and that the dehydrogenase activity may be associated with only part of the protein. Other experiments (not shown) have established that dehydrogenase activity is definitely associated with the 33-kilodalton protein species and possibly with the 43–45-kilodalton species. Thus, peak 5 contains at least one fragment with dehydrogenase activity. Since the apparent molecular weight of the active species is around 40 000, it can be concluded that the active species has a monomeric structure; this fragment is called fragment HDH_M. It has a substantial level of dehydrogenase activity: a rough estimation of the amount of material present in peak 5 indicates that its average specific activity is of the order of 25% of that of native AK I–HDH I.

(*f*) *Peak 6*: *A Monomeric Aspartokinase Fragment, AK_M.* In the experiment shown in Figure 1, the amount of material present in peak 6 was too low to detect the protein(s) on a gel and hardly sufficient to measure its kinase activity. After 60-fold concentration of this material, it is found that this peak contains a single protein corresponding to a molecular weight of 27 000 in the presence of $NaDodSO_4$ (Figure 3c) and possessing a weak but measurable kinase activity. From its elution volume, an apparent molecular weight of 30 000 under native conditions is obtained. This fragment has therefore a monomeric structure and is called AK_M fragment. Its kinase

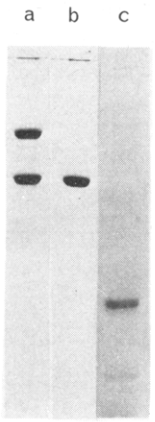

FIGURE 3: $NaDodSO_4$ gel electrophoresis of the fragments. A 60-μL sample of H (a) and HDH_D (b) and a 10-μL sample of 60-fold concentrated AK_M (c) were analyzed.

specific activity is very low, probably not more than 1% of that of native AK I–HDH I. Gel electrophoresis of AK_M fragment under native conditions shows that its mobility is very close to that of (one of the) HDH_M fragment(s) and that it can be detected as such only in the early stages of the proteolysis reaction (Figure 2f).

The amount of material present in peak 6 is increased when proteolysis of AK I–HDH I is carried out for a shorter time with twice as much protease. The properties of AK_M can then be studied without a concentration step and are the same as those described above. The fact that AK_M is produced in different amounts depending on the duration of proteolysis suggests that this fragment is rather unstable and that it is further degraded. Also, the finding that the shorter the time of proteolysis, the higher the relative amount of AK_M indicates that this fragment is produced at an early stage of the proteolytic cleavage of AK I–HDH I. In conclusion, it appears that, despite its low amount and its susceptibility to further degradation, AK_M is indeed one of the defined products of the limited proteolysis of AK I–HDH I; it is a monomeric species with a low but measurable kinase activity.

Time Course of Limited Proteolysis of AK I–HDH I. The complex mixture of fragments produced upon limited proteolysis of AK I–HDH I shows that there are several successive cleavages of the polypeptide chain. The various properties of each fragment, such as size of the chain(s), oligomeric or monomeric structure, and enzymatic activity (activities), already suggest that these fragments are not produced at the same time and appear sequentially. So that the order of their successive appearance, i.e., of their precursor–product relationships, could be established, a kinetic study was performed. The mixture of species present at various times of the proteolytic reaction was analyzed by gel electrophoresis following the presence of protein under native and denaturing conditions and the presence of dehydrogenase activity. Such an analysis is complicated by the fact that the various species are present in different amounts at the same time and that dilute species can escape detection under conditions where major species are readily observed. This difficulty could be partially circumvented by either using different amounts of material for electrophoresis analysis or by taking advantage of the known properties of the isolated fragments to identify the various bands on the gels.

Species Possessing Dehydrogenase Activity. Four species with dehydrogenase activity have been found: native AK I–HDH I and fragments H, HDH_D, and HDH_M. Figure 4a shows the result of a gel electrophoresis under native conditions

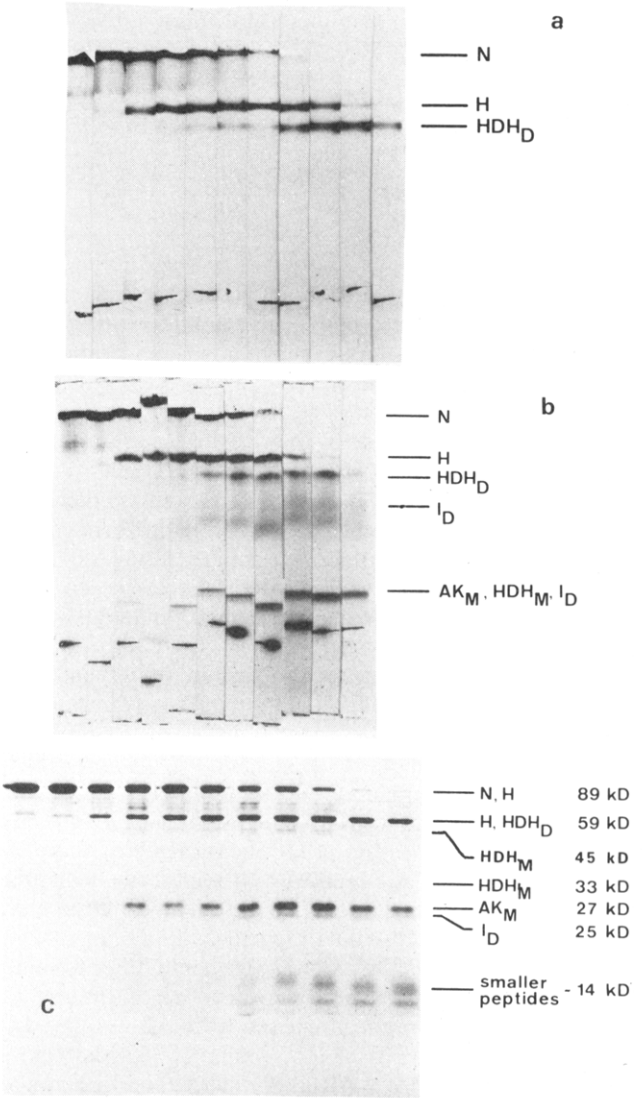

FIGURE 4: Kinetics of the proteolysis of AK I–HDH I. Proteolysis was carried out as described under Materials and Methods [0.5% protease:AK I–HDH I (w/w)]. Aliquots were removed at specified time intervals and 25–30 μg of the protein mixture was analyzed by polyacrylamide gel electrophoresis. HDH activity was revealed in (a). In (b), the gels were stained for proteins. The bands designated as N, H, HDH_D, I_D, HDH_M, and AK_M refer respectively to the native enzyme, the hybrid, HDH dimeric, the inactive dimeric fragment, monomeric HDH, and monomeric AK. In (c), the samples were analyzed in the presence of $NaDodSO_4$. The reaction time is indicated in minutes from left to right on the abscissa and indicates zero time without and with protease, 5, 10, 15, 25, 40, 80, 110, 180, and 210 min, respectively.

with the various species present at different times of proteolysis. It is clear that, as native AK I–HDH I disappears, fragment H is produced before HDH_D. Taking the relative intensities of bands as a crude measure of the amount of protein present, it can be also seen that fragment HDH_D continues to appear at a time where native AK I–HDH I is no longer present. The concomitant increase in the HDH_D band and decrease in the H band strongly suggests that H is the precursor of the HDH_D fragment. The order of appearance, AK I–HDH I $\rightarrow$ H $\rightarrow$ HDH_D, is fully consistent with the properties described above for fragments H and HDH_D.

On the gel shown in Figure 4a, the band corresponding to the activity of the HDH_M fragment can hardly be seen because of the much lower amount of this fragment. Although its position is known (Figure 4b), the intensity of the HDH_M band cannot be used to ascertain the mechanism of its production.

Table I: Proteolytic Fragments of AK I–HDH I

peak no.	fragment	enzymatic act.	native M_r	chain(s) M_r	subunit structure
1	AK I–HDH I	kinase + dehydrogenase	360 000	89 000	tetrameric
2	H	kinase + dehydrogenase	170 000	89 000 + 59 000	dimeric
3	HDH_D	dehydrogenase	115 000	59 000	dimeric
4	I_D		50 000	25 000	dimeric
5	HDH_M	dehydrogenase	40 000	33 000	monomeric
6	AK_M	kinase	30 000	27 000	monomeric

It is, however, likely that a fragment possessing dehydrogenase activity can be produced only from a species with the same activity; also, since HDH_M fragment is smaller (chain length and subunit structure) than any other dehydrogenase fragment, it is probably produced last. Therefore, the order derived from the successive appearance of the dehydrogenase fragments must be AK I–HDH I → H → HDH_D → HDH_M.

Different Protein Species. Figure 4b shows a gel performed at various times of proteolysis, where the different protein species present are separated under nondenaturing conditions. It can be seen that the conclusion derived above is confirmed by the order of appearance of fragments H and HDH_D from AK I–HDH I. It can also be noted that one of the first fragments produced migrates at a position corresponding to that of the native fragments HDH_M and AK_M (which cannot be distinguished under these conditions). This fragment appears at the same time as H fragment does and before HDH_D fragment; it is thus produced directly from AK I–HDH I. If this band corresponded to HDH_M fragment, then HDH_M could not be produced by cleavage of HDH_D as suggested above but by direct cleavage of AK I–HDH I. It is more likely that this band corresponds to AK_M fragment, which would therefore be produced from AK I–HDH I together with H. In addition to the early appearance of the AK_M fragment, this possibility is further supported by other arguments. Indeed, the size (30 kilodaltons) of the fragment excised from AK I–HDH I to form one of the subunits of H fragment (59 kilodaltons) is compatible with the size of AK_M, 27 kilodaltons. Furthermore, the study of the enzymatic properties of purified H fragment shows that the ratio between the levels of kinase and dehydrogenase activities is almost 2 times lower for H fragment than for native AK I–HDH I. This suggests that the part removed from the 89-kilodalton chain to produce the 59-kilodalton chain is related to the region involved in kinase activity. In conclusion, it seems then that the most plausible cleavage mechanism of AK I–HDH I produces fragments H and AK_M at the same time.

Figure 4b also shows that the two bands corresponding to the inactive I_D fragment appear late during the proteolysis of AK I–HDH I. Fragment I_D is hardly detectable at a time when HDH_D fragment is already present in a significant amount, which suggests that I_D is produced later than HDH_D. It seems that, together with the appearance of I_D, there is a marked increase in the intensity of the band corresponding to AK_M and HDH_M. As AK_M is produced quite earlier and at a systematically low level, this increase is probably due to the appearance of HDH_M. This interpretation is only tentative, but the gel in Figure 4b and similar experiments suggest that fragments I_D and HDH_M could be produced at a late stage, presumably from HDH_D. In fact, proteolysis of purified HDH_D did lead to the production of HDH_M and I_D, thus confirming this statement (data not shown).

Chains of Different Sizes. Figure 4c shows the result of electrophoresis in the presence of $NaDodSO_4$ performed at various times of proteolysis. This gel can be used to monitor the time course of appearance of the different chain sizes. As

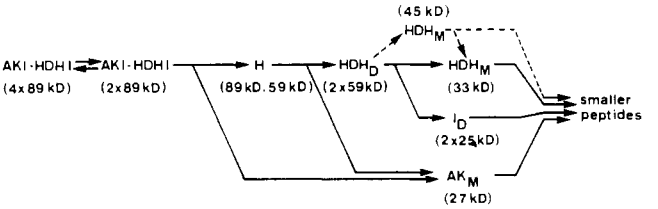

FIGURE 5: Pathway of the proteolysis of AK I–HDH I. The native tetrameric AK I–HDH I is dissociated into a dimer. The dimer is cleaved into the hybrid (H) and the monomeric aspartokinase fragment (AK_M). The hybrid is then proteolyzed to the dimeric homoserine dehydrogenase (HDH_D) and the AK_M. The next step in the proteolysis is the cleavage of HDH_D to produce monomeric homoserine dehydrogenase (HDH_M) and the inactive dimeric fragment (I_D). AK_M, HDH_M, and I_D are further cleaved to give smaller fragments of about 10–12 kilodaltons. The dashed lines indicate a possible minor cleavage of HDH_D.

the 89-kilodaltons band, corresponding to the AK I–HDH I and one of H fragment chains, disappears there is concomitant appearance of the two bands, 59 and 27 kilodaltons. Then, at later times, there is formation of the 45-kilodalton band, followed by that of the 33-kilodalton and 25-kilodalton bands. In the last stages of proteolysis, further degradation takes place, yielding smaller fragments of a defined size 10–12 kilodaltons and thus not occurring at random.

The hybrid nature of H fragment, the heterogeneity of the HDH_M fragments in peak 5, the similarity between the molecular weights of some species, and the different relative amounts of the various fragments make the precise correlation between the formation of a given fragment and the change in chain size sometimes ambiguous. However, the chain sizes found on the separated fragments allow one to make the following assignments: the 89-kilodalton band corresponds to native AK I–HDH I and H fragment, the 59-kilodalton band to H and HDH_D fragments, the 43–45- and 33-kilodalton bands to HDH_M, the 27-kilodalton band to AK_M fragment, and the 25-kilodalton band to I_D fragment. With these plausible assignments, the changes in chain sizes revealed by Figure 4c are in reasonable agreement with the preceding results about the order of appearance of the various proteolytic fragments of AK I–HDH I.

Discussion

Table I summarizes the properties of the various fragments obtained during limited proteolysis of AK I–HDH I. The only tetrameric species is the native uncleaved AK I–HDH I, and any proteolytic degradation is accompanied by the loss of the tetrameric structure. This is in agreement with the known mechanism of proteolysis of AK I–HDH I by subtilisin: the tetrameric structure is not susceptible to subtilisin cleavage and needs to dissociate into a dimeric structure before proteolysis can occur (Véron et al., 1973; Mackall & Neet, 1974). The same is likely to be true here, thus explaining why none of the fragments is able to form a tetrameric structure.

Order of Formation of the Various Fragments during Limited Proteolysis of AK I–HDH I. A tentative scheme of

the succession of events which generates the fragments described in Table I from AK I–HDH I is given in Figure 5. Although this mechanism is not the only one which could account for the present results, it is supported by the following arguments: (a) It is consistent with the monotonic progression expected from a proteolytic degradation, in which simpler structures cannot be the precursors of more complex species. Indeed, in Figure 5, no larger chain is produced from a smaller one, no active species from an inactive one, and no dimeric structure from a monomeric one. (b) It is in agreement with all the results given above about the partial precursor–product relationships between the various fragments. (c) The whole set of proteolytic fragments is generated by the minimum number of cleavages of each polypeptide chain. Indeed, only two cleavages are invoked, with almost no loss of material: the first cleavage would convert a 89-kilodalton chain into a 27-kilodalton chain and a 59-kilodalton chain, and the second cleavage would convert a 59-kilodalton chain into a 33-kilodalton chain and a 25-kilodalton chain. An other cleavage of the 59-kilodalton chain yields the 43–45-kilodalton species and smaller peptides, the former being itself cleaved to the 33-kilodalton chain (see Figure 5). (d) It is in agreement with the postulated three-dimensional geometry of the various globular regions which compose AK I–HDH I (see below). That this mechanism fits the data, satisfies the logic, and shows simplicity makes it attractive, and we will take it as a working hypothesis. The order in which the different fragments are generated from AK I–HDH I has definite implications on the native enzyme structure; this order has much less implications on the individual properties of isolated fragments.

Existence of Monomeric Kinase and Dehydrogenase Fragments. (*a*) *Fragment AK_M.* That kinase activity can be associated with a monomeric species is already known from a nonsense mutant which produces a 53-kilodalton chain (Janin et al., 1967; Vĕron et al., 1972). Although its level of enzymatic activity is low, the AK_M fragment shows that the structural elements necessary for kinase activity correspond to a chain segment of only 27 kilodaltons. This size is compatible with that found for the polypeptide chain of some other kinases, between 20 and 40 kilodaltons (Thuma et al., 1972; Olson & Kuby, 1964). This size for the kinase region of AK I–HDH I also corresponds to that postulated from the nucleotide sequence of the AK I–HDH I structural gene: AK I–HDH I probably results from a gene fusion (Falcoz-Kelly et al., 1972; Vĕron et al., 1972), and possible remains of this fusion are observed as an AUG codon preceded by a ribosome attachment site (Shine & Dalgarno, 1975) at amino acid position 249 (Katinka et al., 1980). The kinase region would thus correspond to the first 248 amino acids, i.e., to a segment of the right size, 27 kilodalton. This result not only supports the size of the postulated kinase region (27 kilodalton) but also assigns it to the N-terminal third of the AK I–HDH I chain.

(*b*) *Fragment HDH_M.* In contrast with the AK_M fragment, it is the first time that a monomeric species possessing dehydrogenase activity is obtained from AK I–HDH I. Even though there might be more than one active fragment, the above results show that the smallest fragment, with a single chain of 33 kilodaltons, is definitely active. Thus 33 kilodaltons is the size of the segment in which the elements involved in dehydrogenase activity are located. This size is compatible with that reported for several NAD-dependent dehydrogenases (Rossmann et al., 1975), including the monomeric octopine dehydrogenase (Olomucki et al., 1972). It is known that the dehydrogenase activity is related to the C-terminal moiety of AK I–HDH I (Vĕron et al., 1972; Sibilli et al., 1981). The assignment of the homoserine dehydrogenase activity to the C-terminal third of the entire polypeptide chain will be developed below.

One of the important observations of this work is thus the existence of two active monomeric fragments of AK I–HDH I. These fragments allow to determine the fraction of each chain which is actually involved in either enzymatic activity. Also, it is likely that the regions corresponding to these segments represent functional domains in the whole native protein, the structure of which is not crucially affected by the absence of either the other chain or part of the chain itself.

Dimeric Dehydrogenase Fragments. Two dimeric fragments possessing dehydrogenase activity have been found, H and HDH_D. These two species are formed upon the same type of cleavage of the polypeptide chain: H is produced by cleavage of the AK I–HDH I dimer in one of the two chains, and HDH_D is produced from H by the same cleavage in the other chain (Figure 5). Proteolysis of purified H indeed leads to the formation of HDH_D and AK_M fragments (data not shown). A cleavage site yielding a 56–57-kilodalton piece from AK I–HDH I has been already found with a variety of proteases (Vĕron et al., 1972; Sibilli et al., 1981), and it is not unexpected to observe cleavage at the same site in the present work. Also, as in the case of other proteases, cleavage at this site on one or the two chains does not abolish the dimeric structure. It can therefore be concluded that the structural elements involved in maintaining this dimeric structure are mainly located on the 59-kilodalton segment (see below).

Besides having dehydrogenase activity, fragment H also possesses kinase activity and threonine inhibition, both being absent in HDH_D fragment. The disappearance of kinase activity upon formation of HDH_D from H can be explained in assigning this activity to the 27-kilodalton segment which is removed. The disappearance of the threonine inhibition of the remaining HDH activity is more puzzling: a detailed analysis of the inhibition kinetics of H is required, but preliminary results show that this inhibition is different from that of native AK I–HDH I (see Results). Therefore, it is expected that a correlation exists between the properties of the various protein species and their regulatory behavior, which involves some specific interactions between the different segments of the polypeptide chains. The results of such studies will be reported elsewhere.

Inactive Fragment I_D. I_D is an inactive dimeric fragment (Table I) which apparently arises from HDH_D (Figure 5). Since I_D is dimeric, it is probably not a degradation product of AK_M or HDH_M; also, since the chain in I_D has the smallest size, it cannot be a precursor of either AK_M or HDH_M. Then, it seems that I_D, AK_M, and HDH_M originate from different regions of the AK I–HDH I chain, i.e., that they are nonoverlapping fragments of the 89-kilodalton original chain. Degradation of HDH_D yields dimeric I_D and monomeric HDH_M; therefore, I_D seems to correspond to a segment of the 59-kilodalton chain which is not involved in the dehydrogenase activity. Rather I_D would correspond to the structural elements implied in some of the contacts responsible for the dimeric nature of HDH_D (and H) and probably for the tetrameric nature of AK I–HDH I.

A tentative location of this 25-kilodalton segment in the AK I–HDH I chain can be proposed, based on the properties of the 53-kilodalton chain extracted from a nonsense mutant. This 53-kilodalton chain has kinase activity, corresponds to the N-terminal moiety of the 89-kilodalton chain, and occurs naturally as a tetramer (Janin et al., 1967; Vĕron et al., 1972). Then if the 25-kilodalton segment of I_D is indeed responsible

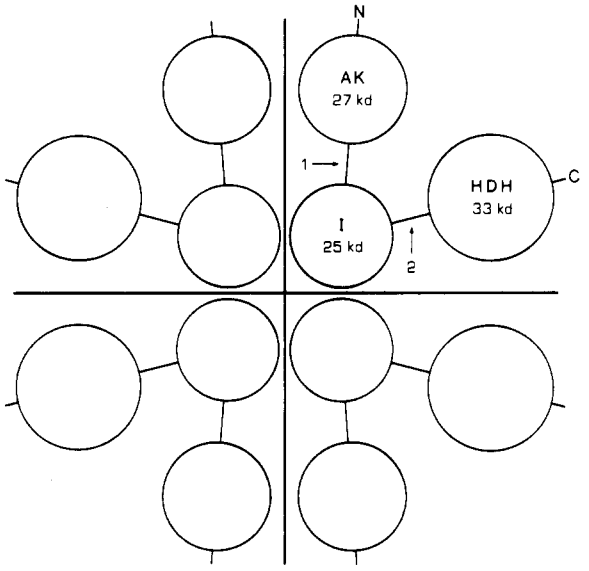

FIGURE 6: Domain I is depicted as responsible for the subunit contacts which generate the dimeric structure of H and HDH_D and possibly the tetrameric structure of the native enzyme. The actual polymeric fragments obtained by limited proteolysis or from extraction from an *ochre* mutant can be visualized as the sum of I and of either of the two catalytically active fragments, yielding the corresponding dimer (HDH_D) or tetramer (AK from the nonsense mutant). The arrows 1 and 2 indicate the sites of the cleavages leading to the different fragments described in the paper.

for interchain interactions, this segment has to be present in this 53-kilodalton chain; I_D would thus correspond to an overlap region between the 53-kilodalton chain and the 59-kilodalton chain of HDH_D, i.e., to the central part of the AK I–HDH I chain. Consequently, the 33-kilodalton segment involved in dehydrogenase activity would be at the C-terminal end of the 59- and 89-kilodalton chains. In conclusion, a tentative assignment of the fragments of the AK I–HDH I chain would be: the 27-kilodalton (AK_M) segment at the N terminus, the 25-kilodalton (I_D) segment in the center, and the 33-kilodalton (HDH_M) segment at the C terminus. The covalent linkage of I_D to HDH_M could be the result of a gene fusion analogous to the one postulated between the regions coding for AK_M and I_D. Indeed, inspection of the sequence of AK I–HDH I (Katinka et al., 1980) shows that, between amino acids 484 and 495, several codons could be transformed by a single mutation into ATG start codons and, preceding them, into nonsense codons; several possibilities exist for ribosome attachment sites at the right distance. If this hypothesis is correct, the corresponding gene product (HDH_M) would be expected to weigh approximately 35 kilodaltons.

A Triglobular Model for the Polypeptide Chain of AK I–HDH I. All the above considerations suggest that the polypeptide chain of AK I–HDH I possesses two preferential cleavage sites and that almost the whole set of proteolytic fragments originates from proteolysis at these sites. The specificity of such cleavages is usually interpreted as showing the existence of compact globular regions. This is true of AK I–HDH I: the 89-kilodalton original chain appears to be cleaved twice, yielding three segments of 27, 25, and 33 kilodaltons (Figure 5). All these three fragments appear to maintain a globular structure when isolated as the products of proteolysis: (a) the 27-kilodalton fragment exhibits enzymatic activity as a monomeric species, AK_M, which shows not only that it is folded but also that its structure is similar to that of the same segment in the whole native enzyme; (b) the 25-kilodalton fragment is able to associate with itself into a dimeric structure, which suggests that it has retained some specific conformation; (c) the 33-kilodalton fragment corresponds to the monomeric dehydrogenase species, HDH_M, and maintains its functional conformation when isolated. Thus, the properties of isolated fragments are consistent with the existence of three compact regions of defined size, in the AK I–HDH I chain; these regions have been tentatively ordered above, and part of their role in the properties of native AK I–HDH I has been found. There is a precedent for such a triglobular structure. It has been shown (Schulze et al., 1978) by X-ray diffraction analysis that the polypeptide chain of the homodimeric glutathione reductase (M_r 50 000) from human erythrocytes is composed of three domains, two being specific ligand binding domains, the dimeric structure being held together by the third one, an interface domain. The sizes, location, and roles of the three segments can be summarized in a model of the intact AK I–HDH I given in Figure 6. These three segments consitute a set of nonoverlapping partition of the intact chain and account for all or almost all of the polypeptide chain. The properties of the different proteolytic fragments, as well as the order of their formation, are in agreement with this model. Moveover, this model can be used as a working hypothesis in interpreting the different aspects of the structure and function of AK I–HDH I and/or its fragments in terms of specific interactions, either between subunits or within a given subunit. Further studies along these lines are now in progress in our laboratory.

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Purification and Characterization of Two Protein Kinases Associated with Rous Sarcoma Virus†

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ABSTRACT: The two major phosvitin-utilizing kinases have been purified from virions of the Prague C strain of Rous sarcoma virus by the use of ion-exchange and affinity chromatography. The two kinases isolated may be differentiated by their molecular weights as well as by their ability to utilize GTP as a phosphate donor. Protein kinase G, which will use either GTP or ATP as a phosphate donor, has a molecular weight of 120 000 as determined under nondenaturing conditions by glycerol gradient centrifugation and 28 000 when assayed under denaturation in sodium dodecyl sulfate (NaDodSO_4)–polyacrylamide gels. Protein kinase A, which will only efficiently use ATP as the phosphate donor, has an apparent molecular weight of 43 000 estimated by glycerol gradient sedimentation and 40 000 by NaDodSO_4–polyacrylamide electrophoresis. Both kinases possess the ability to autophosphorylate. Phosvitin is the major, and casein the minor, phosphate-accepting substrate for both kinases in vitro; however, kinase G will also phosphorylate histones to an extent similar to that observed with casein.

The presence of protein kinases associated with virions of retroviruses has been investigated by a number of groups (Houts et al., 1978; Tsiapolis, 1977a,b; Hizi et al., 1979; Hatanaka et al., 1972; Blaas et al., 1979; Rosok & Watson, 1979). These kinases have varied widely in their molecular weights, in their preference for phosphate donors and acceptors, and in their efficiency of phosphorylation. Three kinases have been identified from the virions of avian retroviruses: a 60 000 molecular weight protein preferring basic phosphate acceptor proteins (Rosok & Watson, 1979), a 45 000 molecular weight protein which utilizes the acidic phosphate acceptors (Fleissner & Tress, 1973), and a 25 000 molecular weight protein that can phosphorylate both acidic and basic phosphate acceptors (Hizi et al., 1979).

The role of the virion-associated kinases has also been investigated. Two reports have indicated that the phosphorylation of reverse transcriptase (Lee et al., 1975), or the phosphorylation of a reverse transcriptase associated protein (Tsiapolis, 1977a,b), can influence the rate of reverse transcription. A later report which investigated the phosphorylation of reverse transcriptase suggested that the β subunit of reverse transcriptase is a phosphoprotein (Hizi & Joklik, 1977). A number of the virion structural proteins have also been observed to be phosphorylated (Fleissner & Tress, 1973; Sen & Todaro, 1977 Leis et al., 1978). Included among these proteins are the two viral structural (gag-related) proteins pp19 and pp12 which are interesting due to their RNA binding capabilities.

The purpose of this investigation was to identify, purify, and characterize those protein kinases associated with Rous sarcoma virus. Futher studies will be initiated using these purified enzymes to specifically investigate their role(s) in the enhancement of reverse transcription by the phosphorylation of the viral polymerase.

Materials and Methods

Chemicals. The proteins used as phosphate acceptors and molecular weight markers as well as ATP and GTP were purchased from Sigma. DEAE-cellulose (DE-52), carboxymethylcellulose (CM-52), and phosphocellulose (PC-11) were purchased from Whatman. Adenosine triphosphate–agarose (ATP–agarose) was from P-L Biochemicals. [γ-^{32}P]ATP and [γ-^{32}P]GTP were prepared in this laboratory by the procedure of Walseth & Johnson (1979). The initial specific activities of the [^{32}P]ATP and [^{32}P]GTP preparations were 6500 Ci/mmol.

Virus. The Prague C strain of Rous sarcoma virus (RSV) was purified from crude virus pellets or ammonium sulfate precipitated virus from supernatants harvested from infected cell cultures. The virus was purified by banding once on a 40% potassium tartrate cushion in 15% sucrose followed by banding twice isopycnically in a 25–50% sucrose gradient (Faras & Dibble, 1975).

Protein and Enzyme Assays. Protein concentrations were determined by the method of either Lowry et al. (1951) or Sedmark & Grossberg (1977). Protein kinase activity was assayed in a buffer of 20 mM $MgCl_2$, 5 mM potassium phosphate, pH 7.5, and 1 mg/mL exogenous protein. For autophosphorylation studies, no exogenous protein was added. One unit of protein kinase activity was defined as that amount of enzyme required to transfer 1×10^{-12} mol of [γ-^{32}P]ATP

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