Critical Role of Asparagine 1065 of Human α2-Macroglobulin in Formation and Reactivity of the Thiol Ester*

(Received for publication, June 27, 1997, and in revised form, September 25, 1997)

Scott A. Suda, Klavs Dolmer‡, and Peter G. W. Gettins§
From the Department of Biochemistry and Molecular Biology, University of Illinois at Chicago, Chicago, Illinois 60612-4316

It has been shown that the relative reaction preference of the C4 thiol ester toward oxygen and nitrogen nucleophiles upon activation by proteinase depends on whether residue 1106 is aspartate or histidine (Dodd, A. W., Ren, X.-D., Willis, A. C., and Law, S. K. A. (1996) Nature 379, 177–179). To determine if the equivalent residue in the related thiol ester-containing protein human α2-macroglobulin (α2M), asparagine 1065, plays a similar role, we have expressed and characterized four α2M variants in which this asparagine has been replaced by aspartate, alanine, histidine, or lysine. The change from asparagine resulted in an altered ability to form the thiol ester. This ranged from failure to form the thiol ester (Asn → Asp) to a maximum extent of formation of about 50% (Asn → Ala). For the three variants that were able to form the thiol ester, the rates of thiol ester cleavage by a given amine were found to be different from one another and slower in nearly all cases than plasma α2M, but with the same relative reactivity of ethylamine > ethylamine + ammonia. The rate of conformational change that follows cleavage of thiol esters in a functional half-molecule was also found to differ between the variants and to be slower than plasma α2M. TNS emission spectra indicated that the conformations of the transformed variants differed measurably from transformed plasma α2M. These findings suggest that residue 1065 plays a critical role in human α2M, for formation of the thiol ester, for its subsequent reaction with nucleophiles, and for the conformational change induced by this reaction. By analogy with C4, where this residue influences the nucleophile preference through direct interaction with the thiol ester, residue 1065 in α2M is expected to be located in or very close to the thiol ester region in α2M.

The abundant human plasma protein α2-macroglobulin (α2M)1 shares a number of properties with the two complement proteins C3 and C4. All three proteins arose from a common ancestral gene (1) and contain a reactive internal thiol ester that becomes even more reactive toward nucleophiles following limited proteolytic cleavage of the protein. In the case of the monomeric proteins C3 and C4, the proteolytic cleavage occurs as part of complement activation. The consequent activation of the thiol ester results in a fraction (~10%) of the activated C3 or C4 forming covalent cross-links to nucleophiles in the vicinity. C3 shows a preference for oxygen nucleophiles (2), whereas C4 shows a preference for nitrogen nucleophiles, but with an increase in reactivity toward oxygen for the B isotype (3, 4). In the case of human α2M, activation of the thiol ester toward nucleophiles and its subsequent cleavage results in a major conformational change of the protein that traps, and thereby inhibits, the proteinase that caused the activation (5).

Given the key role that the thiol ester plays in the functioning of both the two complement proteins and of α2M, it is important to understand both how the thiol ester is formed and what determines its reactivity toward nucleophiles. For α1 inhibitor 3, a rodent protein of the α-macroglobulin family, it has been shown that thiol ester formation is very dependent on the conformation of the protein and only occurs after folding and subsequent conformational rearrangement that involves a disulfide isomerization (6). Similarly, time-dependent conformation-specific reformation of the thiol ester from the amine-cleaved residues that initially formed it has been demonstrated for C3, C4, and α2M (7–9), although without an elucidation of the specific structural requirements for the reformation. More definitive information on the molecular basis for the nucleophile preference of the thiol ester in C4 has been provided by an examination of the effect of single site mutations at position 1106 of C4. Although this is a position 115 residues C-terminal from the cysteine that is involved in forming the thiol ester, it is one of only four residues, occurring in a hexapeptide, that differ between the C4A and C4B isotypes of C4. Law and co-workers (10) showed that histidine at this position (C4B) resulted in enhanced reactivity toward oxygen, whereas aspartate (C4A) resulted in overwhelming preference for reaction with nitrogen nucleophiles. The molecular basis for this was elegantly demonstrated to be the formation of an acyl-histidine intermediate in C4B upon activation, that had enhanced reactivity toward oxygen nucleophiles (11).

The equivalent residue in human α2M is asparagine 1065, which occurs in the hexapeptide SGSSLN. Comparison of all available sequences of α-macroglobulins (13 total) showed that asparagine occurs in 11 and histidine in 2 (Table I). Because of this high conservation of asparagine and the presence of histidine as the only alternative, as in C4B, we sought to determine whether this residue also influenced the properties of the thiol ester in human α2M. Four variants of human α2M, in which residue 1065 was changed from asparagine to alanine, aspartate, histidine, or lysine, were therefore expressed in a baby hamster kidney cell expression system (12), and their properties were determined. We report here the characterization of

1 The abbreviations used are: α2M, α2-macroglobulin; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); TNS, 6-(p-toluidino)-2-naphthalenesulfonic acid; TNB, 2-nitro-5-thiobenzoate.


Table I

| Protein     | Sequence | Source |
|-------------|----------|--------|
| C4A (human) | PCPVLID  | Ref. 26|
| C4B (human) | LSPVH    | Ref. 26|
| C3 (human)  | DAPVH    | Ref. 27|
| α₂M (human) | SGSLLN   | Refs. 28 and 29 |
| α₂M (mouse)| SGVLN    | Ref. 30 |
| α₂M (rat)   | SGSLLN   | Ref. 31 |
| α₂M (rat)   | SGSLLN   | Ref. 32 |
| α₂M (3 rat) | SGSLLN   | Ref. 33 |
| α₂M (Linus) | IGPN     | Ref. 34 |
| α₂M (guinea pig) | SGSLLN | Ref. 35 |
| α₂M (Lamprey) | VQRFLN  | GenBank™ D13567 |
| Meningocoglobin 1 (mouse) | SGSLFN | Ref. 36 |
| Meningocoglobin 2 (mouse) | SGSLFH | Ref. 37 |
| Meningocoglobin (guinea pig) | SOTLHF | Ref. 38 |
| Ovostatin (chicken) | TGLYLN | Ref. 39 |

* Ref. 26
* Ref. 37
* Ref. 30
* Refs. 28 and 29
* Ref. 31
* Ref. 32
* Ref. 33
* Ref. 34
* Ref. 35
* Ref. 36
* Ref. 37
* Ref. 38
* Ref. 39

| Source     | |
|------------||
| Ref. 34    | |
| Ref. 27    | |
| Ref. 38    | |
| Ref. 36    | |
| Ref. 31    | |
| Ref. 32    | |
| Ref. 33    | |
| Ref. 34    | |
| Ref. 35    | |
| Ref. 36    | |

**MATERIALS AND METHODS**

Creation and Expression of α₂M Variants at Position 1065—Plasmid p1167 (8.76 kilobases) (13), a generous gift from Dr. Esper Boel (Novo Nordisk), contains the cDNA for human α₂M under the control of the adenovirus 2 major late promoter. Single site mutagenesis was carried out directly on the intact plasmid using the polymerase chain reaction-based QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). Complementary pairs of oligonucleotides were used. The sequences for the antisense oligonucleotides were 5'-CTT TAT GGC ATT XXX GAG CAG TGA CCC-3', where XXX was GCC for the mutation to alanine, GTC for the mutation to aspartate, GTG for the mutation to histidine, and CTT for the mutation to lysine. The mutations were confirmed by sequencing of a stretch of at least 40 nucleotides centered on the codon for residue 1065. Baby hamster kidney cells were transfected with plasmids containing cDNA for α₂M, dihydrofolate reductase (pSVdhfr), and *Escherichia coli* amino glycoside 3'-phosphotransferase (pEMH140). Methotrexate and neomycin were used to select for stable transfecants, as described (12). Stable transfecants were grown to confluence in roller bottles and cycled between serum-containing and serum-free medium.

**RESULTS**

Lower Than Expected Thiol Ester Content of 1065 Variants—An immediate demonstration that residue 1065 influences thiol ester function in human α₂M was the presence of higher electrophoretic mobility species (fast form) for the variants on a polyacrylamide gel run under nondenaturing conditions (Fig. 1B, odd numbered lanes), indicative of some species without thiol esters (20). The N1065D variant was an extreme case with 100% fast form, indicating no thiol esters. The other three variants showed mixtures of slow, intermediate, and fast electrophoretic forms, with the fast form component ranging from ~53% for the N1065K variant through ~42% for the N1065H variant to a low of ~33% for the N1065A variant, as estimated from scanning of the Coomassie-stained gel and integration. The effect of reaction of each of these samples with methylamine confirmed that the slow and intermediate forms represented a species containing intact thiol esters (full or partial complement, respectively) and the fast form a species lacking intact thiol esters (Fig. 1B, even numbered lanes). Thus, any slow or intermediate form was converted to the fast form through cleavage of the thiol ester by methylamine, whereas the fast form species was unaffected, since there were no thiol esters to be cleaved.

The heterogeneity of the variant species was shown not to be due to the conditions used for production of the BHK-derived recombinant α₂M (Fig. 1A). Thus, both recombinant wild-type α₂M and a “revertant” wild type, in which we had converted the cDNA of the N1065K variant back to wild-type α₂M and expressed the resulting wild-type α₂M, showed a single slow form species with the same mobility as plasma α₂M (Fig. 1A, odd numbered lanes). These two recombinant wild-type α₂M were also completely transformed to the fast form by reaction with methylamine (Fig. 1A, even numbered lanes).
chains cleaved in the bait region. The overwhelming appearance
Brilliant Blue.

A more quantitative estimate of the stoichiometry of thiol esters was obtained from DTNB assay of each of the variants upon complete reaction with methylamine, measuring the appearance of new thiol groups, as described under “Materials and Methods.” The quantitation of thiol esters was based upon the change in absorbance during the reaction and therefore did not include any free cysteine already present in the variants. Based on this assay, the N1065D variant contained no thiol esters, whereas the N1065A, N1065H, and N1065K variants contained 1.9, 1.6, and 1.7 thiol esters, respectively, based on at least five separate determinations and with an uncertainty of no more that ±0.2 thiol esters. Recombinant wild-type αM showed the presence of 3.5 ± 0.3 thiol esters.

**Figure 1.** Incomplete thiol ester formation in αM variants indicated by the presence of significant percentages of fast and intermediate form species on polyacrylamide gel run under nondenaturing conditions and conversion of slow form fraction to fast form by reaction with methylamine. Panel A compares the behavior of plasma αM with that of recombinant wild-type αM and a “revertant” wild-type αM generated by reversing the mutation of the N1065K variant. Panel B compares the behavior of plasma αM with that of the four N1065 variants. Lanes 1 and 2, plasma αM (Pl); lanes 3 and 4, N1065A (A); lanes 5 and 6, N1065D (D); lanes 7 and 8, N1065H (H); lanes 9 and 10, N1065K (K). Under the conditions used for running the gel, plasma αM with four intact thiol esters migrates with slow mobility (lane 1), whereas αM with no intact thiol esters migrates with fast mobility (lane 2). The two recombinant wild-type αM's show identical behavior (lanes 3–6). For both panel A and B, odd numbered lanes contain αM's as purified, and even numbered lanes are αM's reacted with 0.1 M methylamine at pH 8.0 for 2 h to cleave any thiol esters present. Each lane contains 2 μg of αM. Protein bands were visualized by staining with Coomassie Brilliant Blue.

**Figure 2.** Demonstration that fast form variant species did not arise from reaction with proteinase prior to purification from near exclusive presence of intact 180-kDa bands on SDS-PAGE run under reducing conditions. In addition, each αM was incubated with a 2.1:1 ratio of trypsin (based on concentration of trypsin active sites) at room temperature for 5 min, followed by inactivation of the proteinase by the addition of phenylmethylsulfonyl fluoride to a final concentration of 0.5 mM to demonstrate specific bait region cleavage, giving 90-kDa bands. Lanes 1 and 2, plasma αM (Pl); lanes 3 and 4, N1065A (A); lanes 5 and 6, N1065D (D); lanes 7 and 8, N1065H (H); lanes 9 and 10, N1065K (K). Odd numbered lanes, containing αM's prior to reaction with trypsin, contain only 180-kDa bands. After reaction with proteinase, these chains can be cleaved to extents dependent on what fraction of the αM is initially in the slow conformational form (even numbered lanes). Protein bands were visualized by staining with Coomassie Brilliant Blue.
ammonia-treated

One of the characteristic properties of
mentation—

variant.
explains the presence of a fraction of cleavage for the N1065D
region remains somewhat accessible to proteinase (14). This

to uncleaved subunits as was seen for plasma αM with 25 mM amine at pH 9.0 for 20–40 s before dilution into the assay buffer
containing 50 μM TNS.

thiol ester and results in its rapid cleavage. It should be noted
that even in methylamine-produced fast form αM, the bait
region remains somewhat accessible to proteinase (14). This
explains the presence of a fraction of cleavage for the N1065D
variant.

Normal Ability of Slow Form Variants to Undergo Heat Frag-
mentation—One of the characteristic properties of αM, C3, and
C4 is that, when heated, each undergoes autolysis of the
peptide backbone at the glutamate that forms the thiol ester.
This is thought to result from attack of the peptide nitrogen
on the carbonyl of the thiol ester followed by hydrolysis. Such
autolysis therefore requires an intact thiol ester and the ap-
propriate positioning of the thiol ester group and the interven-
ing peptide backbone. To determine if the three thiol ester-
containing variants behaved normally in this regard, samples
were heated to 95 °C for 35 min, and the products were exam-
ined by SDS-polyacrylamide gel electrophoresis. For all three
variants, the characteristic heat fragmentation bands of ~120
and 60 kDa were observed in about the same amounts relative
to uncleaved subunits as was seen for plasma αM (data not
shown), suggesting that residue 1065 is not involved in
this process. The N1065D variant showed no heat fragmentation.

Conformation of Variants in Their “Fast Form” Conforma-

tion Probed by TNS—TNS, noncovalently bound to αM, is a
sensitive monitor of conformational change induced by meth-
ylamine treatment or reaction with proteinase (21–23). αM
has a single class of relatively weak binding sites for TNS (Kd
= 100–200 μM) (23) that changes both affinity and environ-
ment upon change of conformation (21, 23). For human plasma
αM, this results in a large fluorescence enhancement and a
blue shift from 450 to 410 nm upon reaction with either meth-
ylamine or proteinase. We therefore used TNS fluorescence to
compare the conformations of the four variants. Since the
N1065D variant was entirely in fast form, with residue 1065 as
a glutamine, and the other three variants were mixtures of slow,
intermediate, and fast, we first converted N1065A, N1065H, and
N1065K completely into the fast form by reaction with ammonia (for consistency, the N1065D was also treated
with ammonia, although no thiol esters were initially present
and thus no further reaction was possible). The TNS emission
spectra of fast forms of N1065A, N1065H, and N1065K were
almost identical (Fig. 3B), differing only slightly in wavelength
maximum, which was centered at about 430 nm. The spectrum
for N1065D, however, showed a much lower intensity, although
with similar wavelength maximum. In contrast, the spectrum
for ammonia-treated plasma αM showed a very different po-
sition for the wavelength maximum and a lower fluorescence
intensity. Because of the major differences between plasma
αM and the variants and the less dramatic, but reproducible,
differences between the variants, we also compared the spectra
of amine-cleaved plasma αM for cleavage by ammonia and
methylamine, since this would result in different chemical
groups becoming part of the environment of the thiol ester site.
This comparison showed that even the small differences in
these groups have significant effects on the TNS spectra (Fig. 3A),
with both the intensity and wavelength maximum chang-
ing for cleavage by ammonia or methylamine. This suggests
that the TNS binding site either is very close to the thiol ester
site or is exquisitely sensitive to even minor conformational
differences in αM.

Kinetics of Thiol Ester Cleavage by Different Amines—The
second order rate constants for cleavage of the thiol esters in
the three variants that contain some thiol esters were deter-
mined by DTNB assay carried out under pseudo-first order
conditions for ammonia, methylamine, and ethylamine and
compared with the rate constants for plasma αM determined
under identical conditions (Table II). Reactivity toward Tris
was also examined, since the low pKb of 8.08 at 25 °C (24)
enabled high concentrations of the free base form to be
achieved at relatively low total amine concentration. Reactivity
with Tris was so low as to be unmeasurable, even using a final
Tris concentration of 0.5 mM. Each of the three variants showed

\[
\begin{array}{|c|c|c|c|}
\hline
αM & k_a (M^{-1} s^{-1}) & k_b (s^{-1}) \\
\hline
\text{NH}_3 & 0.012 & 0.051 \\
\text{CH}_3\text{NH}_2 & 0.012 & 0.051 \\
\text{C}_2\text{H}_5\text{NH}_2 & 0.012 & 0.051 \\
\hline
\end{array}
\]
the same rank order of reactivity as is found for plasma \( \alpha_M \), namely methylamine > ethylamine > ammonia, with approximately the same relative values for the rate constants, suggesting that accessibility to the thiol ester in each variant is very similar to that in plasma \( \alpha_M \). The mutations at position 1065 did, however, have an adverse effect in nearly all cases on the actual rate constants (Table II). Only histidine was able to confer similar reactivity to plasma \( \alpha_M \) for two of the amines studied, ammonia and ethylamine, although the rate of reaction with methylamine was slower with this variant than with plasma \( \alpha_M \). The magnitude of the rate reduction did not correlate with the size of the replacement residue, as might result if accessibility were being affected, since it was greatest for the smallest replacement, alanine.

Effect of Mutations on the Kinetics of Conformational Change—The major conformational change(s) induced in \( \alpha_M \) by thiol ester cleavage occur cooperatively only after both thiol esters within a half-molecule have been cleaved. To determine whether the mutations at residue 1065 had affected the rate of this conformational change, we determined the rate constant for this step for each of the three variants that contains slow or intermediate form species and for plasma \( \alpha_M \) under identical conditions. From knowledge of the rate of thiol ester cleavage by amines (see above), we calculated that, by incubating the \( \alpha_M \) at very high methylamine concentration at pH 9.0 for 20–60 s, >95% of the thiol esters could be cleaved. By then diluting the sample into the TNS assay buffer, the kinetics of the conformational change step alone could be followed from the change in TNS fluorescence. We have previously used the same approach for determining this rate constant in plasma \( \alpha_M \) after chemical modification of the thiol group (12). The changes in TNS fluorescence showed simple monoexponential behavior, with a rate constant that did not depend on the length of prereaction with methylamine, as long as sufficient time of reaction had been allowed to give nearly complete cleavage of the thiol esters. The rate constants were different both from one another and from the rate constant for plasma \( \alpha_M \) under the same conditions (Table II). As was found for most of the second order rate constants for thiol ester cleavage, the rate constants for conformational change were slower than for plasma \( \alpha_M \), again by a maximum of about 4-fold.

DISCUSSION

We set out to determine whether residue 1065 of human \( \alpha_M \) plays a role in the reactions of the mechanistically important thiol ester by creating four \( \alpha_M \) variants with single site mutations at this position and characterizing the properties of the expressed proteins. The choice of residue 1065 was suggested by the demonstrated importance of residue 1106 of human C4 in the mechanism of thiol ester cleavage (10, 11) and the equivalent positions represented by these two residues based on sequence alignment of the two related proteins (1, 10). We found that residue 1065 of human \( \alpha_M \) not only influenced the reactivity of the thiol ester but also greatly affected the ability of the thiol ester to be formed in the first place. Residue 1065 also influenced the kinetics of the conformational change that results from thiol ester cleavage and resulted in conformations of the fast form of the \( \alpha_M \) variant that were significantly different from those of fast form plasma \( \alpha_M \), as detected by TNS fluorescence emission spectra. For the kinetics of thiol ester cleavage, it was found that the native asparagine at position 1065 gave the fastest rates when compared with the two variants of alanine or lysine and comparable rates for two of the amines with the histidine variant. For the rate of conformational change, asparagine gave the fastest rate compared with the three variants. Together, these alterations in the properties of human \( \alpha_M \) resulting from a single site mutation at position 1065 indicate that the wild-type asparagine at this position is very important for achieving the correct conformation of the protein to ensure thiol ester formation and is probably close enough to the thiol ester, once formed, to ensure optimal reactivity toward nucleophiles, probably through direct interaction with the thiol ester. This presumably accounts for the nearly complete conservation of this residue in the 13 macroglobulins sequenced (Table I).

Although the sequence CGEQ is conserved in macroglobulins, in C4, and in C3 as the tetrapeptide that gives rise to the thiol ester (cysteine and glutamine underlined), there is a body of evidence to support the need for additional specific residues (25) and protein conformations in the vicinity of the thiol ester-forming residues for efficient formation or reformation of the thiol ester in these proteins. The requirement for a specific conformation is presumably to bring about the correct constellation of residues to align and activate the thiol and glutaminyl carbonyl for mutual interaction. The requirement for specific flanking residues has been nicely demonstrated in C3 by site-directed mutagenesis (25) and explains in part why the occurrence of the sequence CGEQ, which occurs in some other unrelated proteins, is not sufficient to bring about thiol ester formation. In rat \( \alpha_1 \) inhibitor 3, it has been shown by pulse-chase experiments that the thiol ester forms only after the folded protein undergoes a gross conformational change that follows slow formation of an interdomain disulfide (6). In human \( \alpha_2 \)M, rat \( \alpha_1 \) inhibitor 3, and complement proteins C4 and C3, it has been shown that it is possible to reform a fraction of the thiol esters following cleavage by ammonia or methylamine (7–9). For reformation of the thiol ester in C3, this is only possible from a transient intermediate conformation (8). Our present findings on the TNS-detected conformational differences between the variants and the different abilities of the variants to form thiol esters are in keeping with these previous results. Thus, the TNS binding sites of the fast forms of N1065A, N1065H, and N1065K variants are all very similar, but they are different from that of plasma \( \alpha_M \) in both wavelength of maximum emission and fluorescence intensity. Each of these three variants forms thiol esters, but less efficiently than wild-type \( \alpha_M \). The N1065D variant has a TNS binding site qualitatively similar to the other variants, based on wavelength maximum of fluorescence emission, but quantitatively different in intensity of the fluorescence, probably reflecting a weaker affinity for TNS. This is paralleled by an inability to form the thiol ester at all. Since alanine, histidine, and lysine have little in common, it suggests that the wild-type asparagine is required for a specific interaction with the thiol ester-forming residues, such as hydrogen bonding to the glutaminyl carbonyl, that occurs less effectively or not at all with other replacements. It should be noted, however, that different classes of these proteins may bring about this activation in different ways. Thus, in C3 the known sequences (human, mouse, chicken, guinea pig, trout, Xenopus, lamprey, sea urchin, and cobra) all have histidine at the position corresponding to 1065 in human \( \alpha_M \).

Thiol ester reactivity toward different nucleophiles has only been examined in detail for human \( \alpha_M \) (19). It has been shown that the rate of cleavage of the thiol ester by nitrogen nucleophiles is a function of the nucleophilicity of the nitrogen and, most importantly, the size of the side chain substituent. The importance of size of the side chain is presumed to result from a relatively restricted access of nucleophiles to the thiol ester in the native state. Together these factors result in a nucleophile reactivity for human \( \alpha_M \) of methylamine > ethylamine > ammonia, with branched chain amines such as Tris or dimethylamine very much less reactive than any of these (19). This is
the same order of reactivity found here for all three of the variants that form thiol esters, suggesting that access to the thiol ester is not affected in the variants by the substitution at position 1065. However, the lower reactivity in nearly all cases again suggests an important role for asparagine 1065 in promoting attack on the carbonyl, which is not as well replicated by alanine, histidine, or lysine. Since histidine and lysine are more effective than alanine, with histidine as effective as asparagine for reaction with ammonia and ethylamine, this suggests that the role of residue 1065 may be to hydrogen bond to the thiol ester carbonyl and make the carbonyl carbon more electrophilic. In this context, it should be realized that the thiol ester-forming residues despite being over 100 residues away in the primary structure. It plays an important role in determining the reactivity of these thiol ester-forming residues both for the initial formation of the thiol ester and in its reactivity to nucleophiles once formed. There are thus parallels with the earlier studies on C4 that prompted the present study, but there are also additional effects that were not seen in C4 that suggest that each of the families of \( \alpha \) macroglobulins and complement proteins may use different constellations of residues to tailor the properties of the thiol ester to the requirements of the protein.

Acknowledgments—We thank Mark Bowen for helpful suggestions, Dr. Steven Olson (University of Illinois at Chicago) for the use of a SLM8000 spectrophotometer and for critical comments on the manuscript, and Dr. Esper Boel (Novo Nordisk) for the generous gift of the \( \alpha_2M \) plasmid p1167.

REFERENCES

1. Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Lønbald, P. B., Jones, C. M., Wierzbicki, D. M., Magnussen, S., Domdey, P. B., Wetsel, R. A., Lundwall, A., Tack, B. F., and Fey, G. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 9–13
2. Law, S. K. A., Minich, T. M., and Levine, R. P. (1984) Biochemistry 23, 3267–3272
3. Isenman, D. R., and Young, J. R. (1984) J. Immunol 132, 3019–3027
4. Law, S. K. A., Dodds, A. W., and Porter, R. R. (1984) EMBO J. 3, 1819–1823
5. Barrett, A. J., and Starkey, P. M. (1973) Biochem. J. 133, 709–724
6. Wasa, M., Enard, F., and Frus, S. (1995) J. Biol. Chem. 270, 24598–24603
7. Grøn, H., Thagesen, I. B., Enghild, J. J., and Pizzo, S. V. (1996) Biochem. J. 318, 539–545
8. Pangburn, M. K. (1992) J. Biol. Chem. 267, 8584–8590
9. Pangburn, M. K. (1992) FEBS Lett. 308, 280–282
10. Sepp, A., Dodds, A. W., Anderson, M. J., Campbell, R. D., Willis, A. C., and Law, S. K. A. (1993) Protein Sci. 2, 706–716
11. Dodds, A. W., Ren, X. D., Willis, A. C., and Law, S. K. A. (1996) Nature 379, 177–179
12. Gettins, P. G. W., Hahn, K., and Crews, B. C. (1995) J. Biol. Chem. 270, 14160–14167
13. Boel, E., Kristensen, T., Petersen, C. M., Mortensen, S. B., Gliemann, J., and Sottrup-Jensen, L. (1990) Biochemistry 29, 4081–4087
14. Gettins, P. G. W., Boel, E., and Crews, B. C. (1994) FEBS Lett. 339, 276–280
15. Hall, P. K., and Roberts, R. C. (1978) Biochem. J. 17, 27–38
16. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
19. Larsson, L.-J., and Björk, I. (1984) Biochemistry 23, 2562–2570
20. Barrett, A. J., Brown, M. A., and Sayers, C. A. (1979) Biochem. J. 181, 411–418
21. Strickland, D. K., Bhattacharya, P., and Olson, S. T. (1984) Biochemistry 23, 3115–3124
22. Björk, I., Lindblom, T., and Lindahl, P. (1985) Biochemistry 24, 2653–2660
23. Strickland, D. K., Larsson, L.-J., Neuenschwander, D. E., and Björk, I. (1991) Biochemistry 30, 2797–2803
24. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) Data for Biochemical Research, p. 424, Oxford University Press, Oxford
25. Isaac, L., and Isenman, D. E. (1992) J. Biol. Chem. 267, 10982–10989
26. Belt, K. T., Carroll, M. C., and Porter, R. R. (1994) Cell 76, 907–914
27. de Bruijn, M. H., and Fey, G. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 708–712
28. Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lønbald, P. B., Magnusson, S., and Petersen, T. E. (1984) J. Biol. Chem. 259, 8318–8327
29. Kan, C.-C., Solomon, E., Belt, K. T., Chain, A. C., and Fey, G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 2282–2286
30. Devriendt, K., Van den Berghe, H., Cassiman, J.-J., and Marynen, P. (1991) Biochim. Biophys. Acta 1088, 95–103
31. Van Leuven, F., Torrekens, S., Overbergh, L., Lorent, K., de Strooper, B., and Van den Berghe, H. (1992) Eur. J. Biochem. 210, 319–327
32. Warmegard, B., Martin, N., and Johansson, S. (1992) Biochemistry 31, 2346–2352
33. Gehring, M. R., Shiels, B. R., Northemmann, W., de Bruijn, M. H. L., Kan, C.-C., Chain, A. C., Noonan, D. J., and Fey, G. H. (1987) J. Biol. Chem. 262, 446–454
34. Bracci, T. A., Norhemmann, W., Hudson, G. O., Shiels, B. R., Gehring, M. R., and Fey, G. H. (1988) J. Biol. Chem. 263, 3999–4012
35. Iwasaki, H., Suzuki, Y., and Sinohara, H. (1996) J. Biochem. 120, 262, 446–454
36. Iwasaki, H., Suzuki, Y., and Sinohara, H. (1996) J. Biochem. 120, 262, 446–454
37. Overbergh, L., Torrekens, S., Van Leuven, F., and Van den Berghe, H. (1991) J. Biol. Chem. 266, 16695–16910
38. Nielsen, K. L., Sottrup-Jensen, L., Nagase, H., and Etzerodt, M. (1994) Ann. N. Y. Acad. Sci. 737, 476–479